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# THE BIOCHEMICAL JOURNAL

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# OBITUARY NOTICE[1]

## BENJAMIN MOORE

(1867–1922)

BENJAMIN MOORE devoted his maturer years to Biochemistry and came to occupy with distinction two important Chairs founded to advance that subject. His early training however was intended to prepare him for the calling of an engineer. A developing taste for pure science, and especially for experimental physics, led him to alter his choice of a career, while the influence of Wilhelm Ostwald a little later directed his mind more particularly to the rapidly growing subject of physical chemistry. The application of physico-chemical methods to biological problems especially attracted him, and he discovered finally that in this sphere was his real vocation.

Although his earlier experimental studies touched in turn very many isolated aspects of physiology and biochemistry, in his latest years he became rather impatient of detail. He determined to deal, so far as might be possible, with the more fundamental problems of biology. He speculated boldly, and believed in the courageous application of experimental methods to the solution even of such great problems as the origin of life. He was indeed always preoccupied with the broader aspect of things; with the riddle of the physical universe as a whole; with the present and future welfare of the human race as a whole. If as a scientific worker he sometimes overvalued the experimental materials on which his bold conceptions were based, he nevertheless often proved himself—when his enthusiasms were less involved—to be an acute critic, alike of his own work and that of others. Many of his papers reveal flashes of real inspiration, and, particularly when discussing physico-chemical aspects of the living cell, he often incidentally advanced ideas which, while wholly original at the time, have since been confirmed experimentally and accepted by others. The progress of biochemistry during the first twenty years of this century owed much to his stimulating publications and to his personal influence over his colleagues and pupils. To the exact data of science his contributions—save in certain limited fields—were not numerous. He lacked the patience, or, perhaps, the stolidity of mind which makes attractive the laborious details of research.

Moore was born in 1867. He was educated at Queen's College, Belfast, now the Queen's University, where he graduated as a Bachelor of Engineering. He then proceeded to Leipzig and worked in Ostwald's laboratory, carrying out a research on the velocity of crystallisation in super-cooled phenol. From Leipzig he went to University College, London, to study physiology under Professor (now Sir Edward) Sharpey Schafer. He was associated with Schafer in researches upon the spleen and the salivary glands, and from the University College laboratories he published work, carried out alone or in conjunction

[1] This notice is based, by the kind permission of the Council of the Royal Society, on the Obituary Notice by the same author, which appeared in the *Proceedings of the Royal Society*, 1927, B. 101, xvii.

with juniors, upon emulsification, upon the chemistry of the suprarenal cortex, and upon the pharmacology of piperidine, coniine and nicotine. To the first volume of Schafer's *Text Book of Physiology* which appeared in 1899, he contributed a critical and very admirable section on the chemistry of digestion, which corrected current errors and stimulated fresh researches. From University College he went to Yale to occupy the post of Associate Professor of Physiology, and from there, conjointly with pupils, he published work upon the origin of lactose, further researches into the function of the suprarenals, and an important paper upon the osmotic properties of colloidal solutions, a subject to which he afterwards paid much attention. After a relatively brief stay in the United States he returned to England and became Lecturer on Physiology at the Medical School of Charing Cross Hospital. During this period of his career he became himself medically qualified. In 1902 he was elected to the Johnston Chair of Biochemistry at the University of Liverpool and at once became very active in prosecuting and stimulating research in his subject. His labours while holding this chair covered a very wide ground. He published, chiefly in conjunction with colleagues and pupils, a quick succession of papers dealing with such diverse subjects as the acid-base equilibrium in the blood of patients with malignant disease, the action of anaesthetics, the action of light and of varying oxygen tensions upon micro-organisms, the oxidising mechanisms of the tissues, the antineuritic vitamin, the toxic action of metals, the phenomena of haemolysis, and other matters. Of his researches carried out at Liverpool those which bore upon the equilibrium between the living cell and its environment seem to have been the most important to his own lines of thought. Three papers: one published conjointly with H. E. Roaf, on the measurement of osmotic pressure in colloid solutions (1907), with another on the osmotic equilibrium of red blood corpuscles (1908), and a third, with A. D. Bigland, dealing with the nature of colloidal adsorption (1911), together contain the chief experimental basis for his thought and teaching concerning the above fundamental subject. He rejected the view that a membrane with specific permeabilities determines the heterogeneous equilibria which obtain between the cell interior and surrounding media. He attributed them rather to the formation within the cell of reversible chemico-adsorptive associations between colloids and crystalloids, involving probably the activity of residual valencies. In the first of the three papers just mentioned Moore put forward many suggestive and enlightened views. It is clear, for instance, that he possessed at this time a fairly definite conception of the membrane equilibrium which, four years later, was quantitatively studied and clearly defined by Donnan.

The great activity of Moore's laboratory after he went to Liverpool yielded much material for publication, and it was sometimes difficult to get papers published without distressing delay. Moore harboured for some time the project of founding a Journal devoted to biochemistry, and finally the generous aid, financial and personal, afforded by Edward Whitley made possible the projected enterprise. Its outcome was the appearance of this *Journal*.

The *Biochemical Journal* was founded in 1906, and until 1912, when it was transferred to the Biochemical Society, it was edited by Moore and Whitley conjointly. No one familiar with the services rendered by the *Journal* will underrate the debt which English biochemists owe to its founders.

In 1911 Moore published a small book called *The Dawn of the Health Age* which contained suggestions for a State Medical Service. He was always much concerned with the welfare of the poor, and this book closes with the following paragraph:

We are one species, we human kind, and one class cannot safely or permanently rise or fall by itself; we must take the others up, or be dragged down ourselves along with them.

In 1912 he was elected a Fellow of the Royal Society. In 1914 he left the Liverpool Chair to join the staff of the National Institute for Medical Research (now so called) at Hampstead under the control of the Medical Research Council. The immediate advent of the war diverted his energies, as those of all his new colleagues, into special channels. He was engaged, for instance, in intensive studies of T.N.T. poisoning and Miners' Phthisis. His views concerning their prevention and cure did not always conform to those of the majority, and he displayed a characteristic fighting spirit in defending them; always with the welfare of the workers in mind.

In 1920 he was called to the Chair of Biochemistry at Oxford founded on the initiative of his intimate friend and former colleague, Mr Whitley. Relieved from the preoccupations of the war, he had turned eagerly to pure science, and when he went to Oxford his mind was full of the problem of the origin of life. His experimental work became an intensive endeavour to throw light upon it. The work he accomplished upon these lines is at least highly interesting, though some of the experiments involved—for instance, those which he believed brought proof that marine and fresh water algae can fix atmospheric nitrogen—were not above criticism. Had he lived, however, his fearless enthusiasm and energy might have carried him far, even on this ambitious quest. He died on May 3, 1922, from sequelae following on an attack of influenza. Lack of health for some little time before had weakened his resistance.

Only a year before his death he published a book on Biochemistry. This is in no sense a general textbook. It deals in the main only with those aspects of the subject which had special interest for its author. It is indeed essentially a summary of his own work, thought and views. It is fortunate that he lived to write it, for it makes clear the outlook to which he had attained and is the scientific testament of an exceptional and most interesting personality.

Moore's Irish origin made him a rebel against orthodoxy and authority, in science no less than in social affairs. It doubtless also helped to make him an inspiring teacher, a delightful colleague, and a most warm and faithful friend.

F. G. H.

# I. A NEW TEST FOR ERGOTHIONEINE UPON WHICH IS BASED A METHOD FOR ITS ESTIMATION IN SIMPLE SOLUTION AND IN BLOOD-FILTRATES.

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(*Received September 13th, 1927.*)

## Introduction.

Tanret [1909] isolated from ergot of rye a base which he termed ergothioneine, which was shown by Barger and Ewins [1911] to be the betaine of thiolhistidine. There is no further record of the occurrence of ergothioneine in the plant or animal kingdom until the isolation of a substance from blood by Hunter and Eagles [1925, 1927], which has recently been shown by Eagles and Johnson [1927] to be identical with ergothioneine. The isolation of a similar substance from blood was also reported by Benedict, Newton and Behre [1926] and that substance has also been shown to be ergothioneine [Eagles and Johnson, 1927; Newton, Benedict and Dakin, 1927].

Two delicate colour tests for ergothioneine have been discovered in the course of recent work in this laboratory. One of these is that ergothioneine gives a blue colour in alkaline solution with the special phosphotungstic acid reagent used for the estimation of uric acid, but the test is not as specific as might be desired, as both uric acid and reduced glutathione also give a blue colour with this reagent. With suitable fractionation of tissue extracts however this test may yet prove of value for quantitative purposes.

The new test to be described for ergothioneine is a special modification of the well-known diazo-reaction.

## Standards and solutions for the test.

1. *Ergothioneine standard.* An aqueous solution of ergothioneine saturated with chloroform and containing 1 mg. in 1 cc. remains unchanged for at least one month if stored in an ice-chest. From this, convenient standards may be prepared containing 0·005–0·050 mg. in 2 cc.

2. *Artificial standard of phenolsulphonephthalein.* (*Phenol red*[1].)

(*a*) *Stock.* 0·10 g. of pure vacuum-dried phenol red is dissolved in 5·7 cc. *M*/20 NaOH in a 100 cc. volumetric flask and water added to the mark.

(*b*) *Standard.* 0·40 cc. of the stock solution is measured into a 100 cc. volumetric flask and diluted to the 100 cc. mark with a buffer solution at $p_H$ 8·0, consisting of 50 cc. *M*/5 $H_3BO_3$ in *M*/5 KCl, and 3·97 cc. *M*/5 NaOH diluted to 200 cc. with water, according to Clark [1920].

The colour of this artificial standard is indistinguishable in daylight in a Duboscq colorimeter from that developed in the test to be described. A test solution (see below) having the same depth of colour as this standard contains 0·015 mg. of ergothioneine.

3. *The diazo-reagent* is made as follows. In a 50 cc. volumetric flask, immersed in running water under 10°, or cooled by ice, are placed 1·5 cc. of a solution containing 9 g. sulphanilic acid and 90 cc. of 37 % hydrochloric acid per litre, followed by 1·5 cc. of 5 % sodium nitrite. The mixture is allowed to stand for 5 minutes, then a further 6 cc. of the nitrite solution is added. At the end of another 5 minutes cold water is added to the mark and the contents are mixed. When kept cold the reagent is good for 2–3 days, but the development of any tinge of colour is an indication that a new reagent should be made.

4. *Sodium carbonate in sodium acetate.* 1 g. anhydrous sodium carbonate and 10 g. anhydrous sodium acetate made up to 100 cc. with water.

5. 10 *N* *sodium hydroxide*, from which carbonate has been allowed to settle.

THE TEST AS PERFORMED FOR QUALITATIVE AND QUANTITATIVE PURPOSES.

1 cc. of the diazo-reagent is delivered into a test-tube. The time is noted, then 0·5 cc. of the carbonate-acetate solution is added, followed after 15 seconds by 2 cc. of the solution to be tested. The contents of the tube are mixed and kept cold. A clear yellow colour develops at this stage. Thirty seconds after the test portion has been added, 2 cc. of the 10 *N* NaOH are added and the mixture is quickly shaken. A beautiful red colour with a purple tinge rapidly develops and is at a maximum after 15 minutes. The colour is stable for at least 40 minutes but on standing for several hours or overnight a purple-red precipitate forms. Very weak solutions fade after 12–24 hours, often without formation of a precipitate, and even a heavy precipitate dissolves with decomposition on longer standing in the strongly alkaline medium. A perceptible colour is obtained with 0·001 mg. ergothioneine in a volume of 5·5 cc.; the test is therefore sensitive to a dilution of 1 in 5,000,000 of ergothioneine.

[1] A very pure product was obtained from Messrs Hynson, Westcott and Dunning, Baltimore. It left no residue on ignition but it was not completely soluble in dilute sodium hydroxide. Some was accordingly purified by dissolving in alkali, filtering and reprecipitating with hydrochloric acid. The precipitate was washed free from sodium chloride and dried to constant weight *in vacuo*. The solution made up from the purified material gave a just perceptibly greater colour by the colorimeter than a corresponding solution made from the original product. The necessity for pure dyes for colorimetric purposes has already been emphasised [Hunter, 1925, 1].

### Remarks on the test.

The characteristic colour of the test is obtained only by the use of strong alkali; sodium or potassium carbonate, sodium cyanide or ammonium hydroxide fails to give the colour. The red colour is changed to yellow on acidification, but returns again on making alkaline with sodium hydroxide. The purplish red precipitate obtained on standing is insoluble in water and in the common organic solvents. Its colour is changed by acids but returns again on making alkaline. An investigation of the chemical nature of this precipitate is desirable before any suggestion as to the possible mechanism of the test can be made.

The test, however, apparently depends on two main reactions. The first of these is the very rapid interaction, or coupling, of ergothioneine with the sodium salt of diazotised sulphanilic acid, with the production of the substance giving the bright yellow colour to the solution. This reaction product of ergothioneine and the diazonium compound is very unstable in an excess of weak alkali, and the second reaction, the formation of a highly coloured sodium salt in the presence of sodium hydroxide, depends on the addition of the latter under conditions where complete coupling has taken place, and at the same time, where a minimum destruction of the coupled product has occurred. The conditions of the test have thus been made as far as possible to meet those requirements.

Thus, it is assumed that the solution to be tested is neutral or very faintly acid, as the quantity of sodium carbonate recommended for use with 1 cc. of the diazo-reagent is just sufficient to leave the solution faintly alkaline after the test portion has been added. The use of sodium acetate along with the carbonate serves to buffer the slight excess of acid in blood-filtrates and leads to the production of both stronger and purer colours than sodium carbonate alone. As the alkalinity of the coupling medium is increased the instability of the coupled product also increases. Thus, for example, if 2·5 cc. of 1 % sodium carbonate are used with 1 cc. of the diazo-reagent, the ergothioneine solution added, and the mixture allowed to stand for 5 minutes, as is the usual technique when testing for iminazoles, then no red colour is obtained on the subsequent addition of strong alkali. The restriction of the coupling time to 30 seconds has the further advantage that it greatly enhances the specificity of the test for ergothioneine by the elimination of various potentially interfering substances. Among these are the iminazoles, especially carnosine, which occurs in blood and tissue extracts [Hunter, 1925, 2], and other substances such as tyrosine and the "acetone bodies." Aromatic phenols, however, couple much more rapidly and at a lower alkalinity than do the iminazoles and acetone, so that phenolic preservatives should be avoided in material to be tested for ergothioneine. Apart from these potentially interfering substances the test has a very high degree of specificity for ergothioneine. No other substance, although many have been examined, has so far been found to give the test.

In certain blood-filtrates in which the concentration of ergothioneine is very low, or the carnosine content is high, a characteristic colour may not develop. Whether the test is positive or negative may however be decided by allowing the mixture to stand overnight, when the formation of even a very slight purplish precipitate indicates the presence of ergothioneine. This precipitate appears to form at greater dilutions of ergothioneine in blood-filtrates than in corresponding simple solutions of ergothioneine, so that for qualitative purposes this aspect of the test is of importance.

On standing with the strong alkali even very dilute test solutions of ergothioneine or blood-filtrates smell of trimethylamine.

### Relationships of the test.

The present test for ergothioneine was discovered when investigating a test obtained in uraemic sera by Andrewes [1924], who found that when the Van den Bergh test was applied to such sera, a yellow or buff colour developed after standing for 24 hours, when, on addition of strong alkali a red colour developed. From the fact that relatively strong solutions of ergothioneine couple in very faintly acid solutions to give a yellow colour the writer at first suspected that ergothioneine accounted for the test described by Andrewes. Subsequent work, however, with uraemic sera has now convinced him that the substance there giving the diazo-reaction is not ergothioneine.

A reaction of a different type, but also given by relatively strong solutions of ergothioneine, has been recorded by Leimdörfer [1924] as given by a substance which he obtained apparently only in small quantity from the blood. Leimdörfer's substance on micro-analysis gave the relationship C 3·5, N 1, H 7–9 which is not sufficiently near to that of these elements in ergothioneine ($C_9H_{15}O_2N_3S$) to establish an identity, especially as sulphur is also said to be absent from Leimdörfer's substance. Nevertheless, it appears very probable that Leimdörfer's base is ergothioneine, a supposition resting almost entirely on the report that when mixed with Ehrlich's diazo-reagent made alkaline with strong alkali a dark red colour slowly develops on standing at ordinary temperature, and more quickly on warming the solution. On long standing a reddish violet precipitate is formed.

It is of importance to note that the thioliminazole ring of ergothioneine does not behave towards the diazo-reagent as does the unsubstituted iminazole ring in such substances as histidine. With the Pauly method, only a yellow colour is developed from ergothioneine, a colour indeed not more red than that given by phenol. Of course, in the Pauly method the reagents in themselves develop a yellowish orange colour on standing for 5–10 minutes and this fact may account for the statement of Barger and Ewins that ergothioneine gives a red colour with this test. This point was of some significance in identifying ergothioneine prepared from blood with that prepared from ergot, and, through the kindness of Prof. Treat B. Johnson, of Yale University, the

writer has had the opportunity of testing ergothioneine prepared from ergot. The substance behaved exactly as ergothioneine prepared from blood.

It was originally stated by Hunter and Eagles [1925] that the substance gives a yellow colour in extreme dilution with the Koessler and Hanke [1919] method. The presence of an unsubstituted iminazole ring in the substance was thus rendered most improbable. When it was discovered, however, that the substance was ergothioneine, the absence of a positive Pauly or Koessler and Hanke test was easily explainable. When the sulphydryl group was removed by ferric chloride with the formation of trimethylhistidine, or, after treatment with strong alkali, by nitric acid to form urocanic acid, according to the methods of Barger and Ewins [1911], then the sulphur-free products in both cases showed the undoubted presence of the iminazole ring by both the Pauly and Koessler and Hanke tests.

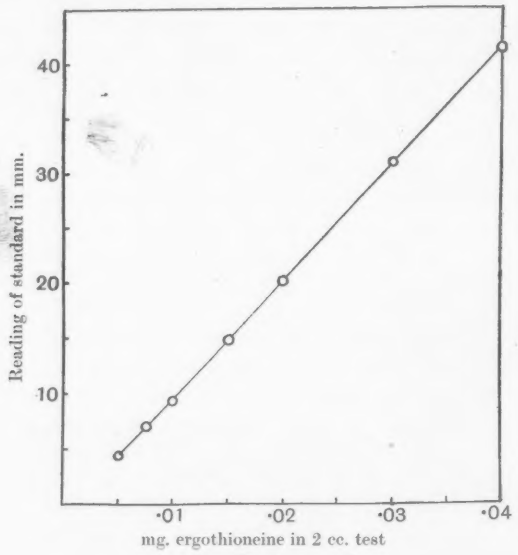


Fig. 1.

QUANTITATIVE APPLICATIONS OF THE TEST.

(*a*) *In simple solutions of ergothioneine.* For quantitative purposes the test is performed as has been described. The colours obtained in test solutions are conveniently matched against standards made from known amounts of ergothioneine, or they may be matched, preferably in daylight, against the phenol red standard described.

The test solution, after the colour has fully developed, is transferred to the left cup of the Duboscq colorimeter and its plunger is set at 15 mm. The right cup is partly filled with the standard and the plunger in this cup is

moved until the colour of the standard matches that given by the test. The readings obtained with the phenol red standard when the test was set constantly at 15 mm., from different concentrations of ergothioneine, in the range of 0·005–0·040 mg., are given in Fig. 1.

It is seen from Fig. 1 that the depth of colour produced in the test solution is a straight line function of the amount of ergothioneine in it. The slope of this line is 1·06 and the number of mg. of ergothioneine in the test solution can be calculated from the equation

$$\text{ergothioneine in mg.} = \frac{\text{reading of standard in mm.} + 0{\cdot}9}{1060}.$$

(*b*) *In blood-filtrates.* Protein-free blood-filtrates made according to the tungstic acid method of Folin and Wu [1919] have been found to be suitable for the test in all types of blood so far examined. By this method whole blood is precipitated at 1 in 10 dilution and corpuscles generally at 1 in 20 dilution. The 2 cc. portion used for the test thus represents 0·2 cc. of whole blood and 0·1 cc. of corpuscles. In many bloods this is a sufficiently large test-portion from which to obtain colours suitable for reading, and indeed in some pig bloods a fifth of those amounts may be sufficient. In many other bloods, however, the 2 cc. test-portion is insufficient to permit of satisfactory readings on account of their low concentration of ergothioneine. By decreasing the dilution in the precipitation of the protein, this difficulty may be overcome.

Estimations of ergothioneine of a preliminary nature have been carried out on a number of bloods from various animals. The values reported in Table I are given as mg. of ergothioneine in 100 cc. of corpuscles as the substance is confined almost entirely to the corpuscles. The highest accuracy is not yet claimed for the quantitative application of the test, especially where the amount of ergothioneine in the blood is less than 5 mg. per 100 cc. But there is no doubt of the substantial correctness of the values given in Table I as may be seen from the following considerations. In many pig blood-filtrates the proportionality of colour obtained in the test is as good as that obtained in simple solutions of ergothioneine. Ergothioneine added under these circumstances is quantitatively determinable by the method. Further evidence will be given on this point in a forthcoming paper. The addition of ergothioneine representing as little as 2 mg. per 100 cc. of blood is qualitatively recognisable by the test, so that the absence of an appreciable colour in ox blood-filtrates is due to the very small amount of ergothioneine in ox bloods and not to the presence of substances inhibiting the development of colour in the test. The amount of aromatic phenols in blood is negligible when the ergothioneine is more than 5 mg. per 100 cc., but below this value the colour given by the ergothioneine is not quite pure, owing probably to the combined interference of small amounts of phenols and iminazoles.

Of the bloods reported in Table I that from pig has an almost constantly high content, those from ox and sheep a constantly low content of ergothioneine. The amount of ergothioneine in blood even of the same species,

however, varies over a wide range. The amount in human corpuscles varies from about 2–10 mg. per 100 cc. No blood so far examined has been found to be entirely free from ergothioneine.

Table I. *Ergothioneine in blood to nearest mg. per* 100 *cc. corpuscles.*

| | | |
|---|---|---|
| Normal men | (9) | 2·5; 3; 5; 8; 3; 7; 5; 3; 4 |
| Pig | (12) | 28; 21; 20; 20; 30; 26; 5; 43; 31; 4; 53; 47 |
| Ox | (10) | All <2 mg. |
| Rabbit | (4) | 5·5; 7; 9; 2 |
| Guinea-pig | (5) | 2; 6; 4·5; 8; 3 |
| Fowl | (12) | 3 to 8. Two over 20 |
| Cat | (2) | <1 |
| Dog | (1) | 5·5 |

Few normal tissue constituents are subject to the variations here indicated for ergothioneine, but it is interesting to note that a similar variation has been recorded for carnosine [Hunter, 1922] which contains, in common with ergothioneine, the iminazole ring.

## Summary.

1. A new and apparently highly specific colour test, depending on the use of diazotised sulphanilic acid, has been described, by which it is possible to detect ergothioneine in a dilution of more than one in five millions.

2. A method for the quantitative estimation of ergothioneine in simple solution and in protein-free blood-filtrates has been based upon the test.

3. The ergothioneine contents of a number of human and animal bloods are recorded.

The writer is indebted to the Medical Research Committee of this University for access to their animals.

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# II. STUDIES ON THE EFFECT OF HEAT ON MILK.

## IV[1]. THE IODINE CONTENT.

By HUGH EDWARD MAGEE and AGNES ELISABETH GLENNIE.

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(*Received November 11th, 1927.*)

Experiments have been reported showing that reduction in the soluble calcium of milk by heat [Magee and Harvey, 1926, 1] (*a*) adversely affects the metabolism of calcium, phosphorus, and nitrogen [Magee and Harvey, 1926, 2], and (*b*) may eventually give rise to gross signs of rickets [Orr *et al.*, 1926] in young animals fed on heated milk. Symptoms other than those resulting from defective formation of bony tissue have been attributed to the feeding of heated milk to children [Hess, 1920]. Cod-liver oil has been quoted [Maddock, 1927] as having improved the growth-promoting properties of heated milk. Cod-liver oil is comparatively rich in iodine [Kelly, 1925]. These considerations suggested an investigation into the effect of heat on the iodine content of milk. As the investigation proceeded the question of the physico-chemical state of the iodine content was raised. Experiments dealing with this problem are also included in this paper.

### *Effect of heat on the total iodine.*

Separated cow's milk ("fresh," "pasteurised" and "boiled") was prepared as before [Magee and Harvey, 1926, 1]. In addition samples were prepared by bringing separated milk in an open beaker rapidly to boiling point and allowing it to cool on the bench ("boiled in open"). By continuous stirring film formation was avoided. Total iodine was estimated in duplicate by the Leitch-Henderson [1926] modification of Fellenberg's method.

Of the 13 samples studied (Table I) only eight were subjected to all the heating processes. While the changes due to heat in all the samples are comparable with one another, only the findings in regard to these eight samples are averaged for the sake of comparison. It is evident that heat brought about considerable losses in the iodine content. The fact that these were greater in the case of the boiled milk than in the case of pasteurised milk seems to indicate a progressive effect of the heat. Further, the relative evenness of the losses in the case of the boiled milk would suggest that the limit of loss which heat could effect had been nearly reached.

[1] No. III of this series appeared in the *Scottish Journal of Agriculture* [Orr *et al.*, 1926] but by accident was not headed as belonging to the series.

Table I. *Iodine content before and after heating.*

| "Fresh" γ per 100 cc. | "Pasteurised" γ per 100 cc. | Loss % | "Boiled" γ per 100 cc. | Loss % | "Boiled in open" γ per 100 cc. | Loss % |
|---|---|---|---|---|---|---|
| 6·00 | 4·00 | 33 | — | — | — | — |
| 6·36 | 4·31 | 32 | — | — | — | — |
| 4·94 | 3·78 | 23 | 3·68 | 25 | — | — |
| 6·30 | 5·15 | 18 | 4·94 | 22 | 4·73 | 25 |
| 4·90 | 3·90 | 20 | 3·60 | 27 | 4·40 | 10 |
| 4·73 | 4·10 | 13 | 3·68 | 22 | 3·89 | 18 |
| Av. 7·46 | Av. 5·36 | Av. 28 | Av. 5·46 | Av. 27 | Av. 5·15 | Av. 31 |
| 5·69 6·90 | 4·51 5·90 | 20 15 | 4·23 5·30 | 26 23 | 4·42 5·70 | 22 17 |
| 5·36 | 3·89 | 27 | 3·47 | 35 | 3·89 | 27 |
| 4·94 | 3·89 | 21 | 3·68 | 25 | 3·47 | 30 |
| 4·94 | 3·89 | 21 | 3·68 | 25 | 4·10 | 17 |
| 4·20 | 3·68 | 12 | 3·26 | 22 | — | — |
| 4·31 | 3·68 | 15 | 3·36 | 22 | — | — |

The disappearance of iodine seemed to be due to volatilisation. To confirm this inference approximately 10 g. of iodine-free KOH per 300 cc. were added to several samples of milk which were prepared as before. Obviously, there was then much more alkali present than was necessary to fix, and therefore to prevent volatilisation of, all the iodine present in the milk.

Table II. *Iodine content before and after heating in presence of KOH.*

| "Fresh" γ per 100 cc. | "Pasteurised" γ per 100 cc. | Change % | "Boiled" γ per 100 cc. | Change % | "Boiled in open" γ per 100 cc. | Change % |
|---|---|---|---|---|---|---|
| 4·20 | 4·00 | −5 | 4·30 | +3 | 4·00 | −5 |
| 3·70 | 3·60 | −3 | 3·80 | +3 | 3·90 | +5 |
| 4·83 | 4·52 | −7 | 5·04 | +4 | 4·83 | 0 |
| 4·52 | 4·20 | −7 | 4·31 | −5 | 4·20 | −7 |
| Av. 4·31 | 4·08 | −5·5 | 4·36 | +1·25 | 4·23 | −1·75 |

The variations found (Table II) in the iodine contents of the heated milks as compared with the raw samples are within the limits of error of the analytical method and are therefore without significance.

*The state of iodine in milk.*

Harvey [1927] has stated that about 80 % of the total iodine in goat's milk is diffusible, but he did not report the experiments in detail. We have, as being more appropriate, included them in this paper, especially because the iodine estimations were all carried out by one of us (A. E. G.). Fresh separated goat's milk only was used.

Ultrafiltration was carried out by Thiessen's apparatus[1]. The membranes used were made by soaking No. 40 Whatman filter papers in a solution containing 2·5 % collodion in glacial acetic acid, allowing them to drip and then washing away all trace of acid in running water. Previous trials had shown that such membranes just held back all the colloids of milk. Pressures of three atmospheres were supplied by means of a simple form of pressure apparatus fitted with a motor tyre valve. Ultrafiltration for 30 hours usually

[1] Supplied by Vereinigung Göttinger Werke, Göttingen.

sufficed to give a perfectly clear ultrafiltrate of about 7 cc. from 10 cc. of milk. Ultrafiltration was always continued until the residue was quite gummy and immobile.

Dialysis was carried out by the method previously described [Magee and Harvey, 1926, 1] but distilled water was used instead of running tap water and all membranes were made from a solution containing 4 % collodion in glacial acetic acid. 20 cc. of milk were put in each sac and three of these at a time were dialysed against 2 litres of distilled water which was changed every 2 hours, the total period of dialysis being 8 hours. The iodine in the contents of each sac was then estimated so that each result is an average of three estimations. The results are shown in Table III.

Table III.

| Ultrafiltration: | | | | | | | Average |
|---|---|---|---|---|---|---|---|
| 1. Iodine in separated milk, $\gamma$ per 100 cc. | 17 | 14 | 22 | 20 | 14 | | 17 |
| 2. Iodine in ultrafiltrate, % of total | 82 | 75 | 75 | 78 | 75 | | 77 |
| 3. Iodine in residue, % of total | 15 | 15 | 17 | 20 | 22 | | 18 |
| 4. Sum (2+3) | 97 | 90 | 92 | 98 | 97 | | 95 |
| Dialysis: | | | | | | | Average |
| Iodine in separated milk, $\gamma$ per 100 cc. 18 | 11 | 13 | 11 | 14 | 12 | | 13 |
| Iodine in residue, % of total 14 | 16 | 15 | 16 | 17 | 18 | | 16 |

The results of the ultrafiltration experiments gave an average of 77 % of the total iodine in the ultrafiltrate and 18 % in the residue, *i.e.* 95 % of the iodine was recovered. This result is satisfactory considering the complicated nature of the technique. By dialysis an average of 16 % was obtained in the residue. This figure is in close agreement with the 18 % found by ultrafiltration. The average of these two results, 17 %, therefore, most probably represents the non-diffusible iodine. That is to say, 83 % of the total iodine of separated goat's milk was found to be diffusible.

*The iodine content of milk-fat.*

The above experiments having been carried out on milk separated by a separator, it seemed of interest to compare the iodine contents of the samples before and after separation. It will be seen (Table III) that the average iodine content of the 11 samples was 15$\gamma$ per 100 cc. of separated milk. Before separation the same samples showed an average content of 17$\gamma$ per 100 cc. of whole milk, *i.e.* a difference of 2$\gamma$. These findings indicate that the fat contained more iodine than an equal volume of separated milk. Most probably the iodine associated with the fat was chemically bound with the unsaturated fats and presumably therefore non-diffusible. The separated milk used in the experiments unquestionably contained some fat, because the most efficient separator, even when set, as in these experiments, so as to remove the maximum of cream, invariably leaves traces of fat behind. It does not, however, seem possible that the presence of such small amounts of fat in the separated milk could account for all the non-diffusible fraction. It seems

therefore, that this fraction for the most part consists of iodine in close, non-dissociable, chemical union with either organic or inorganic colloids or with both. The average fat content of the above 11 samples (of whole milk) as determined by the Soxhlet method was 6·52 g. per 100 cc.

*Effect of heat on the state of iodine.*

Separated cow's milk, raw and heated, was used for these experiments. Three lots of 20 cc. from each sample were dialysed as before and the iodine estimated in the residues.

Table IV.

| "Fresh" milk | | "Pasteurised" milk | | "Boiled" milk | |
|---|---|---|---|---|---|
| $\gamma$ per 100 cc. before dialysis | Loss % by dialysis | $\gamma$ per 100 cc. before dialysis | Loss % by dialysis | $\gamma$ per 100 cc. before dialysis | Loss % by dialysis |
| 6·0 | 79 | 4·0 | 82 | — | — |
| 6·4 | 77 | 4·3 | 78 | 3·7 | 74 |
| 4·9 | 87 | 3·9 | 88 | 3·6 | 88 |
| 4·2 | 88 | 3·9 | 86 | 3·3 | 87 |
| Av. 5·4 | 83 | 4·0 | 84 | 3·5 | 83 |

The results (Table IV) suffice to show that after heating, the diffusible and non-diffusible fractions of the residual iodine bore the same relation to each other as they did in the original raw milk. Further, the data indicate that the relative amount of diffusible iodine in separated cow's milk is approximately the same as in separated goat's milk (83 %).

SUMMARY.

Heat caused the disappearance by volatilisation of 20 % or more, according to the duration of heating, of the total iodine of separated milk. The ratio of diffusible to non-diffusible iodine was the same in the milk after heating as it was before. Approximately 83 % of the total iodine of separated cow's and goat's milk was found to be in diffusible form.

We desire to express our gratitude to Dr D. Harvey for his co-operation in the ultrafiltration experiments, to Dr Isabella Leitch for instruction in the technique of the iodine analysis, and to Dr J. B. Orr for his criticism.

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# III. THE HYDROGEN ION CONCENTRATION OF THE FAECES OF RACHITIC CHILDREN.

By THIRZA REDMAN.

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(*Received November 14th, 1927.*)

The investigation was undertaken as a sequel to work done on rats by Zucker and Matzner [1924], and by Bacharach and Jephcott [1926], in which it was found that the $p_H$ of faeces of rats fed on a rickets-producing diet was consistently alkaline, but that when vitamin D was administered a change occurred in the reaction of the faeces, which gradually became acid and remained so—this change synchronising with the cure of the rachitic condition of the rats and, therefore, suggesting an excellent criterion for determining the clinical condition of the animal with regard to rickets. The importance in regard to rickets in children was at once suggested by these results in rats.

If so simple a method of following the clinical course of the disease could be obtained in rachitic children it would be of much value, especially at clinics where X-ray apparatus was not available. Consequently, the faeces of a number of rachitic children were examined for $p_H$ and the same method used as in previous work [Redman, Willimott and Wokes, 1927], viz. the electrometric method with the quinhydrone electrode.

In the first place, faeces were obtained from rachitic children receiving artificial sunlight treatment (tungsten-cored carbon arc lamps) as out-patients.

Twelve unselected cases which had been treated for different periods, and varying in age from one to three years, were examined.

The following figures were obtained:

Table I.

| Case | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| $p_H$ | 4·74 | 5·90 | 4·64 | 5·42 | 5·25 | 5·94 | 4·21 | 5·08 | 6·74 | 5·61 | 5·72 | 5·70 |

As these were all on the acid side it raised hopes that the children were all being cured and that the hypothesis would be found correct.

These children were all out-patients on varying diets. Twelve hospital cases were next selected that had been admitted as very severe rickets and had been under treatment for a considerable period. They were all on the same standard antirachitic diet. X-ray evidence showed healing in five cases (Nos. 13, 15, 18, 22 and 23) and that the other severe cases were improving, but not healed, at the time of commencing observations.

In Table II are given the details of the different treatments, the time in days taken to establish a cure according to X-ray findings, the number of days after admission to hospital before the first $p_H$ value of the faeces was determined, and, finally, a few $p_H$ values with the number of days' interval between the readings.

Table II.

| No. | Age in years | Treatment | Time taken to cure according to X-ray (days) | Days after admission before $p_H$ taken | 1st $p_H$ | Interval (days) | 2nd $p_H$ | Interval (days) | 3rd $p_H$ | Interval (days) | 4th $p_H$ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 13 | $1\frac{8}{12}$ | CLOM. AST. | 143 | 162 | 4·99 | 16 | 6·17 | 8 | 4·59 | 5 | 4·50 |
| 14 | 4 | CLOM. AST. | 196 | 125 | 6·11 | 20 | 6·80 | 1 | 8·41 | 8 | 5·30 |
| 15 | $1\frac{6}{12}$ | CLOM. AST. | 138 | 216 | 6·02 | 14 | 5·32 | 7 | 8·29 | — | — |
| 16 | 3 | CLOM. AST. and syr. fe. phos. | 257 | 103 | 6·57 | 15 | 6·23 | 7 | 5·04 | 7 | 6·84 |
| 17 | 3 | CLOM. and oleum phos. | 191 | 102 | 5·59 | 15 | 6·05 | 7 | 5·05 | 7 | 4·97 |
| 18 | 2 | CLOM. | 223 | 295 | 5·11 | 14 | 7·49 | 3 | 6·13 | 7 | 5·53 |
| 19 | $2\frac{11}{12}$ | AST., kalzana | 178 | 123 | 6·45 | 16 | 7·36 | 6 | 7·10 | 7 | 8·70 |
| 20 | 2 | CLOM. AST., kalzana | 169 | 124 | 5·76 | 29 | 4·97 | — | — | — | — |
| 21 | 2 | CLOM. AST., thyroid | 174 | 138 | 5·33 | 20 | 6·41 | — | — | — | — |
| 22 | $3\frac{6}{12}$ | AST. | 166 | 182 | 7·28 | 10 | 6·76 | 5 | 6·02 | 1 | 7·54 |
| 23 | 2 | CLOM. AST., kalzana | 102 | 188 | 7·10 | 8 | 7·31 | 6 | 6·00 | 7 | 7·30 |
| 24 | | CLOM. AST., kalzana, thyroid | 216 | 156 | 6·91 | 8 | 7·31 | 7 | 4·68 | 7 | 6·13 |

CLOM. = Cod-liver oil and malt. AST. = Artificial sunlight treatment.

In the first set of reactions, only two were alkaline (Nos. 22 and 23) and these samples were from cured cases. Subsequent sets of reactions showed alkaline values in all cases but three, one of which was cured and two were not cured.

Samples of faeces from seven untreated cases of severe rickets and from two normal children were next examined, with the results shown in Table III.

Table III.

| Cases of rickets | | | | Normal children | |
|---|---|---|---|---|---|
| No. | $p_H$ | No. | $p_H$ | No. | $p_H$ |
| 25 | 7·71 | 29 | 6·13 | 32 | 5·53 |
| 26 | 4·60 | 30 | 5·08 | 33 | 6·49 |
| 27 | 5·61 | 31 | 8·56 | | |
| 28 | 4·59 | | | | |

Only two samples were found to be alkaline, both from cases of rickets. The rest were acid.

So far, no particular construction could be placed upon the reactions of the faeces obtained from untreated cases of rickets or cases that had received treatment in hospital for over 100 days. It was therefore decided to take some really severe cases of rickets that had received no treatment at all and to follow the reaction of the faeces over a number of days, keeping the children

in the hospital and feeding them all on the same standard antirachitic diet, but putting them on different treatments. Fourteen rachitic children (Nos. 34–47), aged 2 to 3 years, were selected, and, together with one normal child as control, were all put on the same diet. Two younger children (Nos. 48 and 49), aged 15 months, suffering from severe rickets were also included but they did not have the mince, chicken, or cocoa supplied to the older children. The diet of the older children consisted of:

| | | |
|---|---|---|
| Milk. | Custard. | Bread. |
| Cream. | Beef tea or chicken broth. | Rusks. |
| Cocoa, made with milk. | Mince. | Butter. |
| Raw egg beaten in milk. | Minced chicken. | Oranges. |
| Blancmange. | Bacon gravy. | |

The different treatments were as follows:

1. Cod-liver oil and malt together with:
   (*a*) Artificial sunlight (tungsten-cored carbon arc).
   (*b*) Sunlight on balcony.
   (*c*) Mercury vapour lamp.
2. Ostelin.
3. "British Drug Houses" irradiated ergosterol.
4. Irradiated ergosterol No. 2.
5. Radio-malt.
6. Irradiated cholesterol freshly prepared every few days.

The children were first kept on the special diet for 5 or 6 days without special treatment.

On the first three days no samples of faeces were collected, to allow as much as possible of the food taken previous to admission to hospital to be swept out of the alimentary canal before beginning the series of observations.

The staff was asked to collect daily as much as possible of the faeces passed in 24 hours and a representative sample was then taken from each case. The $p_H$ was determined daily, first of all for 2 or 3 days before treatment was begun, then during treatment for periods varying from 26 to 49 days, except in a few cases where an interval was left after starting treatment to see if the hypothetical "consistently acid" state would have been reached before taking $p_H$ values. It was not always possible to get the daily samples. Undoubtedly some of the children suffered from constipation, but more often the organisation broke down.

The results are shown graphically in Figs. 1 to 7. There are, of course, the gaps to be taken into account where "constipation" occurred (or, where sometimes the samples were thrown away), but a certain amount of comparison is possible between cases undergoing the same treatment.

Fig. 6 shows the curve obtained in the case of the normal child. On each of the other figures is marked the treatment given and a note of X-ray findings.

From Figs. 1 to 6 no correlation can be inferred between the $p_H$ of the faeces and the condition of the patients with regard to rickets as shown by skiagrams.

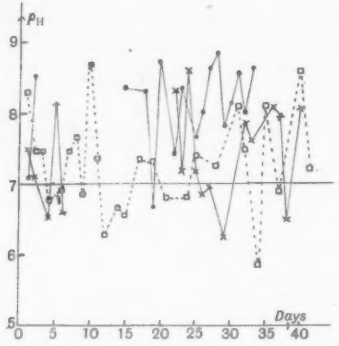


Fig. 1.

| Case | Curve | Treatment |
|---|---|---|
| 34 | ——×—— | Cod-liver oil and AST. |
| 35 | ——•—— | ,, and balcony |
| 37 | - - -□- - - | ,, and Hg vapour lamp |

X-ray, all nearly healed, 40 days

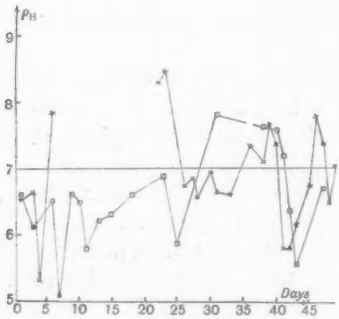


Fig. 2.

| Case | Curve | Treatment | X-ray |
|---|---|---|---|
| 38 | ——×—— | Ostelin | Healed 61 days |
| 39 | ——□—— | ,, | ,, 58 days |

The majority of the readings are markedly on the alkaline side in Fig. 1, but vary enormously from acid to alkaline and back, regardless of the condition of the patients in Figs. 2, 3, 4 and 5. In Fig. 6 the variations in the case of the control child on the same diet are quite as marked as in the cases of rachitic children.

Fig. 7 shows the curves for the two babies on the same diet and treatment, and for one older child on the same treatment, but different diet. It was unfortunate that the older child contracted measles, and observations could not be continued longer, as the reactions of the faeces were more on the

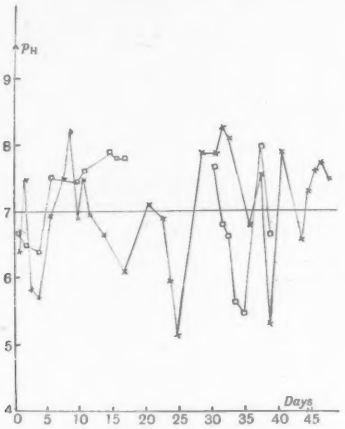


Fig. 3.

| Case | Curve | Treatment | X-ray |
|---|---|---|---|
| 40 | ———×——— | BDH. ergosterol | Nearly cured 63 days |
| 41 | ———□——— | ,, ,, | ,, 32 days |

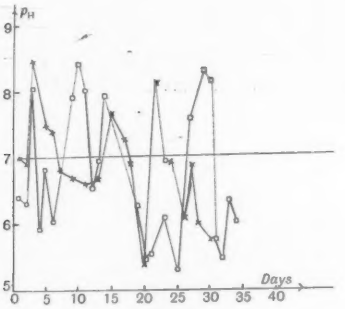


Fig. 4.

| Case | Curve | Treatment | X-ray |
|---|---|---|---|
| 42 | ———×——— | Ergosterol (2) | Healing 30 days |
| 43 | ———□——— | ,, | ,, |

alkaline side than those of the babies. The faeces of one baby varied much more in their reaction than those of the other. The latter was cured more quickly than the former, and its faeces were only once alkaline, but the condition of one on admission was as bad as that of the other.

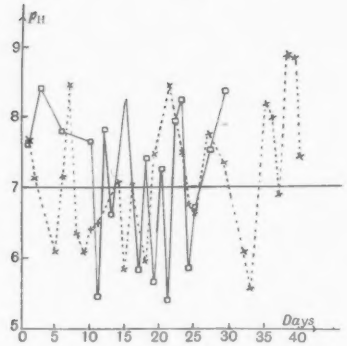


Fig. 5.

| Case | Curve | Treatment | X-ray |
|---|---|---|---|
| 44 | - - - × - - - | Radio-malt | Nearly healed 38 days |
| 45 | ——□—— | ,, | Healing 19 days |

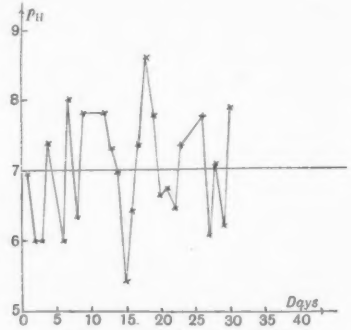


Fig. 6. Normal control child

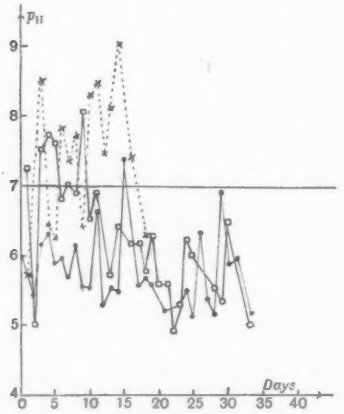


Fig. 7.

| Case | Curve | Treatment | X-ray |
|---|---|---|---|
| 46 | - - - × - - - | Irradiated cholesterol | Improved 19 days |
| 48 | ——•—— | ,, | Healed 35 days |
| 49 | ——□—— | | Nearly healed 36 days |

## Conclusion.

It has not been found possible to obtain a definite correlation between different stages of the disease of rickets and the $p_H$ values of the faeces of the patients, under the conditions described in the paper. The $p_H$ determinations were all made by means of the quinhydrone electrode.

I am deeply indebted to Dr A. Dingwall Fordyce and to Col. Macdiarmid for free access to cases under their care, to Dr Aidin, R.M.O., and the Staff of Alder Hey Hospital for their help and courtesy whilst the experiments were being carried out, and to Prof. Heilbron and Dr Kamm who prepared irradiated cholesterol and ergosterol used for some of the treatments. For part of the apparatus for the electrometric determinations I am indebted to the Royal Society.

The work was done in the Bacteriological Department, Liverpool University, during the tenure of the Virol Research Scholarship in Clinical Pathology.

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# IV. THE ISOLATION OF A SECOND STEROL FROM YEAST-FAT.

## (PRELIMINARY COMMUNICATION.)

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(*Received November 17th, 1927.*)

THE isolation of a sterol from yeast was successfully accomplished by Gérard who obtained it as white crystals melting at 135–136°. Gérard [1895] regarded it as belonging to the same group as the ergosterol (M.P. 154°) prepared by Tanret [1889] from ergot. He showed that they both gave a typical colour reaction with chloroform and sulphuric acid which differentiated them from cholesterol and phytosterol and he regarded them as characteristic of the sterols occurring in the group of cryptogams. Hinsberg and Roos [1903] isolated two sterols from yeast, melting respectively at 159° and 148–149°. They regarded the lower melting sterol as a separate substance since they were unable to raise its melting point by further recrystallisation. Later Neville [1913] isolated only a substance melting at 148–149°.

Working with Thomas [1920] I investigated the various constituents of yeast-fat and obtained a crude sterol melting at 135–136°, which after being many times recrystallised melted in an open tube at 154°. Tanret [1908] found that the melting point was best taken in a sealed capillary tube filled with $CO_2$ as the oxidation products formed by heating ergosterol in air when the determination is made in an open tube lower the melting point. This probably accounts in part for the discrepancies as to melting point which are recorded in the literature.

We worked with a considerable number of samples of brewery yeast: from one of these only no ergosterol was isolated but in its place a white crystalline sterol melting at 97–98° and having a specific rotation of + 10·1° in chloroform solution. So little material was available that we were then unable to examine it further. Since that time very many samples of yeast have been examined and in them all ergosterol has been found. I had however noticed at various times that although it is possible to obtain from yeast-fat relatively good yields of solid white sterol melting somewhere between 120° and 140°, only a comparatively small yield is obtained melting at or above 154°. It has now been found possible to separate this crude sterol, by recrystallisation from alcohol, ether and acetone into (1) the less soluble ergosterol, melting at 158·5° (in an open capillary tube), and (2) a white crystalline sterol which, after many recrystallisations, melted in an open capillary tube

at 108–109°. The two sterols appear to occur in varying proportions: in one preparation a rough estimate based on the amounts of high and low melting fractions separated after repeated recrystallisations indicated that the proportion of ergosterol was about twice that of the lower melting sterol. It will be convenient to refer to the latter as *zymosterol* from its source of origin, until more is known of its relation to ergosterol. Of these two sterols, zymosterol appears to be the more unstable to the action of air and light.

*Optical rotation.* The new zymosterol is dextrorotatory; the specimens originally obtained melted between 99° and 104° and gave in ether solution specific rotations for the green mercury line at 20° of + 16·1°, + 16·5°, and + 16·8°. In chloroform solution one of these specimens gave a specific rotation of + 17·3°. By many subsequent recrystallisations from ether, acetone and a mixture of alcohol and ether, a specimen was obtained melting at 108–109°, and the specific rotation of this in ether solution was + 34·1°. The specimen of ergosterol melting in an open capillary tube at 158·5° gave a specific rotation $[\alpha]_{5461} = -130°$ in chloroform solution. The highest value I can find recorded for the specific rotation of ergosterol is that given by Tanret for the anhydrous substance in chloroform solution, $[\alpha]_D = -132°$.

It is probable that the values for both the melting point and specific rotation here given are too low, for the examination of the absorption spectrum (cp. p. 25) indicates that the zymosterol still contains small amounts of ergosterol, but I have not so far had sufficient material available to continue the recrystallisations until a pure specimen was obtained. From the examination of the absorption spectrum it seems that the amount of ergosterol present must be less than 5%. Since ergosterol is laevo- and zymosterol is dextro-rotatory, the determination of the specific rotation affords the most convenient method of following the separation of a mixture of these two substances.

*Analysis.* A micro-combustion of each of the two sterols was made and gave the following results for the anhydrous substances dried to constant weight in a vacuum desiccator.

| | | |
|---|---|---|
| 5·510 mg. zymosterol: | 5·483 mg. $H_2O$ | H, 11·05 % |
| | 17·124 „ $CO_2$ | C, 84·72 % |
| 4·282 „ ergosterol: | 4·480 „ $H_2O$ | H, 11·62 % |
| | 13·27 „ $CO_2$ | C, 84·54 % |
| Calculated for $C_{27}H_{42}O$ | H, 10·99; | C, 84·17 % |

Like ergosterol, zymosterol is quantitatively precipitated by digitonin. It follows therefore that the estimation of the amount of ergosterol in yeast-fat, by weighing the precipitated digitonide, gives the amount of the two sterols and is not a measure of the ergosterol content.

The *acetate* of zymosterol was prepared by heating 0·78 g. of the sterol with 5 cc. of acetic anhydride and warming on the sand-bath for 15 minutes. After cooling and diluting the mixture, the crystals which separated were

washed with water and recrystallised from alcohol. The crystals first separating melted at 112–113°: after three recrystallisations the melting point was raised to 115°. The acetate crystallises in thin flat plates radiating from a point. The iodine value determined by Hübl's reagent was 226: the calculated value for $C_{27}H_{41}OOC.CH_3$ containing three double bonds is 179·7.

The sterol gives a modified Salkowski colour reaction: when shaken with chloroform and sulphuric acid a yellow colour is produced in the sulphuric acid layer instead of the red brown colour produced in the case of ergosterol.

*The number of ethenoid linkages.* It has previously been pointed out that the determination of the iodine value of ergosterol presents considerable difficulty [Smedley-MacLean and Thomas, 1921]. If Wijs solution is used to determine the iodine value, the numbers obtained are nearly twice as great as those given with Hübl's reagent. Even with the latter the values obtained for the sterols are high and vary with the excess of reagent present, the time during which the reaction mixture stands and its temperature.

From the consideration of the figures obtained for ergosterol, the presence of three ethenoid linkages in the molecule was deduced [Smedley-MacLean and Thomas, 1920], a result which has since been confirmed by Windaus from the behaviour of this sterol on hydrogenation [Windaus and Grosskopf, 1923].

In determining the iodine value of the new dextro-sterol the corresponding number for ergosterol was obtained at the same time under quite similar conditions. Thus, using Hübl's reagent, the values for a specimen of ergosterol (which melted in an open tube at 145°) and for the dextro-sterol (M.P. 96–98°) were respectively 236 and 241·2. The anhydrous sterol $C_{27}H_{42}O$ containing three ethenoid linkages requires 199·4.

Dam [1924] recommends the pyridine sulphate-bromine reagent in glacial acetic solution[1], described by Rosenmund and Kuhnhenn [1923] for the determination of the iodine value of cholesterol, and claims that when this reagent is used constant results are obtained even if considerable variations in the excess of reagent and the time of reaction take place. The method has the advantage that the reaction is ended in from 3 to 5 minutes, and Dam found that no increase in the value for cholesterol occurred even if the time was extended to 15 minutes.

0·02 g. sterol was dissolved in 5 cc. chloroform and 10 cc. of the pyridine reagent were added. After standing for 5 minutes 3·5 cc. of a 10 % solution of potassium iodide were added and the mixture was titrated with $N/10$ thiosulphate until the brown colour had almost disappeared: 1 cc. of starch solution was then added and the titration completed. It may be noted that the chloroform solution becomes deep yellow in colour, so that the starch must be added when the *brown* colour of the iodine is still visible.

The iodine values obtained by this method were, for a sample of ergosterol

[1] The reagent is made by adding 8 g. pyridine to 10 g. concentrated $H_2SO_4$ in 20 cc. glacial acetic acid. A solution of 8 g. bromine in 20 cc. glacial acetic acid is added and the mixture made up with the same solvent to a litre.

(M.P. in open tube, 158·5°), 296·0 and for the zymosterol (melting at 99–101°, $[\alpha]_{5461} = +16{\cdot}1°$), 299·8. If the reagent was left in contact with the sterol solution for 15 minutes, considerably higher values were found and excess of the reagent seemed to produce the same effect. As far as these results go the method seems no more satisfactory than that of Hübl for the estimation of the iodine value of the highly unsaturated sterols.

From these results it is probable that the number of ethenoid linkages in ergosterol is the same as that in the zymosterol now described and further that the structure of both substances must be closely similar, since whatever be the structure other than an ethenoid linkage, which is responsible for the abnormally high iodine values obtained, it is present in both sterols alike.

Zymosterol therefore like ergosterol probably contains three ethenoid linkages.

*The action of bromine.* A well-cooled solution of 0·3 g. bromine in 10 cc. dry ether at 0° was slowly added to a solution of 0·25 g. zymosterol in 12·5 cc. ether. The solution was at first rapidly decolorised. The excess of bromine and ether were removed *in vacuo* and a brown oil remained giving off HBr and turning a deep green. The behaviour on bromination is very similar to that of ergosterol.

*Absorption spectrum in the ultra-violet.* Dr N. S. Lucas very kindly examined for me the absorption spectra in the ultra-violet region of specimens of ergosterol and zymosterol separated from the crude yeast sterol. The specimens were first examined in a dilution in alcohol of 1 in 20,000. At this dilution the characteristic absorption bands of the ergosterol spectrum were visible only in the light transmitted through the ergosterol solution. In that transmitted through the solution of zymosterol there was no evidence of any selective absorption. When the specimen of zymosterol (M.P. 108–109°) was examined in a concentration of 1 in 5000 the absorption bands in the ultra-violet region characteristic of ergosterol became visible. They were however not as marked as in a solution of ergosterol in a concentration of 1 in 100,000. It is therefore probable that in this specimen of zymosterol, ergosterol was present but to a less extent than 5 %. It seems possible that a comparison of the absorption spectra of solutions of these compounds will provide the best method of detecting the presence of ergosterol as an impurity in specimens of zymosterol.

The biological action of the zymosterol is at present being investigated.

## Discussion.

It is evident that two sterols exist in yeast, clearly differentiated by their difference in optical rotation.

The evidence now brought forward supports the view that zymosterol is closely related to ergosterol. Both are precipitated by digitonin; they are equally unsaturated and contain at least three ethenoid linkages. Probably the high iodine values obtained by all the methods employed are to be ascribed to some peculiarity of structure present in the sterols rather than to the

presence of additional ethenoid linkages, for even with cholesterol abnormal values are found.

In Tanret's original work on ergosterol, he isolated from ergot a second sterol melting at 144° (on the Maquenne block) and giving in ether solution a laevorotation $[\alpha]_D = -12{\cdot}9°$. He named this fungisterol and ascribed to it the formula $C_{25}H_{40}O.H_2O$; its acetate melted at 158·5°. I have so far not had an opportunity of comparing zymosterol with fungisterol. The melting points of the latter and of its acetate respectively are about 30° to 40° higher than those of the corresponding zymosterol compounds: on the other hand the specific rotation of fungisterol in ether solution is $[\alpha]_D = -12{\cdot}9°$ compared with $[\alpha]_{5461} = +34{\cdot}1°$ for zymosterol and $[\alpha]_D = -105°$ for ergosterol. While the possibility is not excluded that fungisterol is an impure form of zymosterol, the available evidence does not support this view. A further study of fungisterol is necessary before a definite conclusion can be reached.

At present but little can be said as to the relation of the dextrorotatory zymosterol to the laevorotatory ergosterol. Windaus [1916] showed that the four isomeric dihydrocholesterols, coprosterol, $\beta$-cholestanol, pseudocholesterol and $\epsilon$-cholestanol are all dextrorotatory. In the first two of these the steric position of the hydroxyl groups, each of which is attached to a similarly placed asymmetric carbon atom, is the same and both these sterols are precipitated by digitonin. Pseudocoprosterol and $\epsilon$-cholestanol however are differentiated from coprosterol and $\beta$-cholestanol by the steric position of the hydroxyl group and neither of these is precipitated by digitonin. This may perhaps be regarded as an indication that in ergosterol and zymosterol the steric positions of the OH and H groups are similar.

The best known instances of dextrorotatory sterols previously described are (1) the four dihydrocholesterols referred to above, all of which are saturated, and (2) *iso*-cholesterol, about the structure of which we know very little. The existence of a dextrorotatory and highly unsaturated sterol in yeast is therefore of particular interest.

I desire to acknowledge my indebtedness to the Department of Scientific and Industrial Research for a grant received during the course of this investigation, and to Dr N. S. Lucas for the examinations of the ultra-violet absorption spectra of these substances.

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# V. THE EXAMINATION OF YEAST-FAT FOR THE PRESENCE OF VITAMINS A AND D BEFORE IRRADIATION AND OF VITAMIN D AFTER IRRADIATION.

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In 1924, one of the writers (I.S.-M.) with E. M. Luce carried out some experiments on rats in which the effect of the addition of yeast-fat to a diet deficient in the fat-soluble vitamins was tested [1925]. At the time at which this work was undertaken it was not generally recognised that the fat-soluble vitamin factor contained two components. Although this view was originally brought forward in 1922 by McCollum, Simmonds, Becker and Shipley [1922], it was not till after the publication of a paper by Steenbock and Nelson [1924] that the presence of two distinct vitamins in cod-liver oil was clearly established; a deficiency of one of these (A) was shown to be responsible for the symptoms of ophthalmia and a deficiency of the other (D) was correlated with defective calcification of the bones, while both were able by their absence to limit growth. Since 1924, it has been clearly recognised that both vitamins A and D are present in cod-liver oil but that only the effect of the D vitamin can be replaced by irradiation of the animal. The results obtained by Luce and Smedley-MacLean showed that the addition of yeast-fat to a diet deficient in the fat-soluble vitamins resulted both in increased growth and in increased calcification of the bones; they attributed these results to the presence of the fat-soluble vitamin A in the yeast-fat since at that time the vitamins A and D were not differentiated. In the light of present knowledge the results then obtained would be interpreted as indicating the presence of vitamin D in the unirradiated yeast-fat and as yielding no information as to the presence or absence of vitamin A. The influence of yeast-fat in promoting growth confirmed the conclusions previously drawn by Hopkins [1912] from his experiments in which a marked effect on growth was obtained by the addition of the ether-soluble extract of yeast to a diet deficient in the fat-soluble vitamins. It seemed therefore of interest to repeat the work of Luce and Smedley-MacLean and to extend it to a separate examination for the two vitamins A and D which improved technique has now made possible.

### Examination of yeast-fat for vitamin A.

The test for vitamin A was made by using the method described by Chick and Roscoe [1926]; young rats of about 40 g. weight were placed upon a diet[1] deficient in vitamins A and D and after about 5 weeks on this diet, the hardened cottonseed oil, forming about 10 % of it, was replaced by the same oil which had been irradiated for 30 minutes, using a mercury vapour quartz lamp, thus supplying vitamin D. The rats then began to grow more vigorously but ceased growing again in another two weeks and began to show signs of decline, with symptoms of xerophthalmia and general vitamin A deficiency. At this point 10 drops of yeast-fat, about 0·18 g., were administered to 2 rats and 5 drops to a third rat. The yeast-fat used was prepared by the direct extraction of pressed brewery yeast with ether, just as was the fat used by Luce and Smedley-MacLean in their experiments. The rats disliked it very much but took it fairly well. There was no restoration of growth but a progressive decline of condition with falling weight until the rats had to be killed after from 10 to 20 days of treatment with very severe symptoms of xerophthalmia and vitamin A deficiency (see Fig. 1).

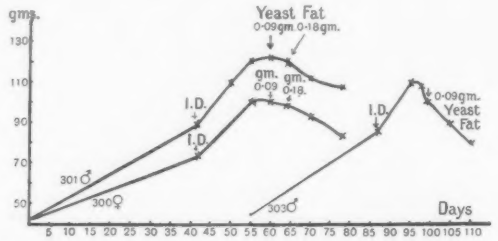


Fig. 1. *Vitamin A in total yeast-fat.* Three rats received a diet deficient in both vitamins A and D up to the point I.D. where a diet deficient in vitamin A only was substituted. When growth ceased a daily dose of 0·09 g. total yeast-fat was administered, which in the case of rats 300 and 301 was increased after several days to 0·18 g.

No resumption of growth occurred and the decline in weight and condition progressed until the rats showed severe symptoms of vitamin A deficiency, and had to be killed.

It would appear therefore that vitamin A is absent from yeast-fat or present in so small a quantity that it cannot be detected in such a dose as it was found possible to administer. The growth induced in rats in the experiment of Luce and Smedley-MacLean, when a daily dose of 0·14 g. fat was administered, prepared similarly by directly extracting dried pressed brewery yeast with ether, was probably therefore not due to the presence of vitamin A as was then supposed but would be accounted for if the fat contained vitamin D.

[1] The diet used was made up as follows: inactivated caseinogen, 300 g.; starch, 750 g.; hardened cottonseed oil, 225 g.; marmite, 75 g.; salt mixture, 75 g.; lemon juice, 75 cc.; distilled water, 900 cc. The salt mixture consisted of sodium chloride, 51·9 parts; magnesium sulphate, 164 parts; sodium dihydrogen phosphate, 104·1 parts; dipotassium hydrogen phosphate, 286·2 parts; calcium phosphate, 162 parts; calcium lactate, 390 parts; ferric citrate, 35·4 parts.

### Examination of yeast-fat for vitamin D.

In testing for the presence of vitamin D, young rats of about 40 g. weight were placed upon the same diet, deficient only in vitamins A and D, as was used in making the test for vitamin A, but *without* the subsequent addition of any irradiated cottonseed oil. Sexes and litters were distributed as evenly as possible among the different experimental groups. After the rats had been for 5 weeks or more on the diet, and at a time when their weight curves were flat, the dose of yeast-fat was administered daily for a period of about 5 weeks, at the end of which time the rats were killed; the bones of both hind legs were then dissected out, dried, extracted with ether and alcohol and the percentage weight of ash determined and compared with the similar values obtained in the controls.

The growth, which takes place during and in response to the administration of the test dose of vitamin D, is taken as some indication of the potency of the given dose, particularly at the beginning of administration. The amount of this growth is, however, also dependent upon the amount of the rat's reserve of vitamin A and it cannot therefore be taken as an accurate criterion. It is, however, very helpful in adjusting the size of the dose in the first instance: if no growth response takes place in the first few days after the beginning of administration of the dose, it can be assumed that the dose is too small and it is then easy to increase it instead, perhaps, of carrying through a large experiment on a whole series of inadequate doses.

When carrying out the test for the detection of vitamin D, a considerable quantity of yeast-fat which had been prepared from yeast, incubated for 48 hours in the glucose-phosphate solution, as described on p. 30 (cp. Specimen I, p. 30), was available. As the supply of yeast-fat prepared by direct extraction of dried pressed brewery yeast which had been used for the examination for vitamin A was now exhausted, it was decided to carry out the tests for vitamin D on the fat derived from the incubated yeast, the sterol content of the two fats being almost identical (see Table I).

A dose of 10 drops (0·14 g.) of the fat was administered daily for some weeks to each of 2 rats but the results, both as regards growth and as regards percentage ash in the dried bone (Table II), were similar to those yielded by the controls, and must be regarded as entirely negative. The fat derived from the incubated yeast, although similar in sterol content, did not behave like the fat used in the previous experiments of Luce and Smedley-MacLean. In the incubated yeast there is a rapid and abnormal conversion of carbohydrate to fat and sterol. As will be seen later, after irradiation the fat obtained from the incubated yeast was as potent in vitamin D as the fat obtained directly from the pressed yeast freshly removed from the wort. Luce and Smedley-MacLean state that they had no evidence of the presence of the fat-soluble vitamin except in the fat derived from freshly pressed yeast as received from the brewery. This would lead to the important deduction

that some condition is present when yeast is growing in wort where a free supply of nitrogenous food is available which can produce the same result as irradiation, though to a much more limited extent. It is proposed to make a comparison of yeast-fat prepared by various methods and to ascertain what are the conditions of its preparation which lead to the presence in it of vitamin D.

### Examination of irradiated yeast-fat for vitamin D.

Since Rosenheim and Webster [1927] have identified the provitamin D as ergosterol itself or as some closely related substance, the investigation of irradiated yeast-fat, which contains this sterol in relatively large amount, is of special interest.

Three different specimens of yeast-fat were used for irradiation; the methods of preparation and characteristic properties of these were as follows.

*Specimen I.* This was prepared from pressed brewery yeast which had been incubated for 48 hours in a well-aerated solution containing 5 % glucose, 0·37 % $Na_2HPO_4$ and 0·029 % $KH_2PO_4$ [Smedley-MacLean and Hoffert, 1924]. The yeast was allowed to settle, filtered off and repeatedly extracted with cold alcohol. The solvent was removed under diminished pressure, the residue dissolved in ether and as the lipins were needed for other experiments, they were removed by precipitation with acetone, and the acetone-soluble fat used for irradiation.

*Specimen II.* This was obtained from the original pressed yeast by repeatedly extracting it with alcohol at the ordinary temperature. It differed from Specimen I in that the yeast had not been incubated in the glucose-phosphate solution before its extraction. Starting with the same quantity of pressed yeast, the fat content is never more than one-fifth of that obtained from yeast which has been incubated. The lipin fraction was not removed from this specimen.

*Specimen III.* This was obtained from another sample of yeast which had been first incubated in the glucose-phosphate solution as in Specimen I. The yeast was then boiled with normal HCl for 2 hours, filtered and the dried residue extracted with ether in a Soxhlet apparatus.

The properties of these three specimens are shown in Table I. The sterol content was estimated by precipitation with digitonin after hydrolysis of the fat.

Table I. *Properties of specimens of yeast-fat.*

| | Iodine value of fat | Unsapon. fraction % | Sterol % | Iodine value of unsapon. fraction |
|---|---|---|---|---|
| Specimen I | 146·5 | 41·7 | 9·7 | 271·4 |
| „ II | 137·6 | 35·2 | 10·2 | 285·3 |
| „ III | 156·5 | 31·3 | 2·65 | 205·4 |

### *Method of irradiation.*

A drop of yeast-fat was weighed on a Petri dish after being spread over as large a surface as possible and exposed for 1 hour to the rays from a mercury vapour quartz lamp at a distance of 32 cm.

*Estimation of dose.*

Having previously determined the sterol content of the fat, the weight of fat which would contain 1/400 mg. of sterol was taken as unit dose and the number of unit doses contained in the drop of fat calculated. Fresh fat was irradiated each day. The fat was then dissolved in glycerol, the quantity of glycerol added being such that one drop of the glycerol solution of fat would contain the unit dose of sterol.

In one experiment the unit dose was reduced to one-fifth of that originally taken by further dilution with glycerol. In this case the dose of sterol actually given was 1/2000 mg. Controls and rats receiving less than the maximum dose of fat and glycerol received corresponding amounts of pure glycerol only. The work of Rosenheim and Webster [1927] has shown that ergosterol after irradiation with ultra-violet light is extraordinarily potent in preventing the onset of rachitic symptoms in rats fed on a diet deficient in the fat-soluble vitamins, the minimum dose of the irradiated sterol necessary being from 1/10,000 to 1/20,000 mg. or even less. In estimating the dose of fat which would be equivalent to this dose of activated sterol, two facts have to be borne in mind.

(1) Yeast contains two sterols, both of which are precipitated by digitonin [Smedley-MacLean, 1928]. One of these, ergosterol, becomes active when irradiated: the biological behaviour of the other, zymosterol, is at present under investigation. The amount of sterol in yeast, as estimated by precipitation with digitonin, does not therefore represent the amount of ergosterol present.

(2) So far experiments have not been carried out to determine the most favourable conditions for irradiation. It is possible that a product of greater activity might be obtained by irradiating the fat in solution in a suitable organic solvent. In the case of the sterols irradiation of the solids does not give satisfactory results and it is quite possible that the irradiation of the highly pigmented yeast-fat is not as satisfactory as irradiation in a suitable solvent, a point that requires further investigation.

*Biological tests with irradiated yeast-fat.*

The biological test for vitamin D was carried out on the irradiated fat in the same manner as that on the non-irradiated fat described above (p. 29).

*Specimen I.* Doses of 0·005, 0·025 and 0·125 mg. of the fat were administered to groups of 3 rats each and the results compared with those given by controls (see Tables II and III). The two larger doses gave results which must be regarded as the same; both of them show a very high percentage ash. The dose of 0·005 mg. produced some increase in ash content over that shown by controls of the same sex and litter, but the controls are themselves rather high and a dose of 0·005 mg. would appear to be approaching the minimum dose of this particular sample of irradiated yeast-fat.

Table II.

A. *Showing percentage ash in the dried extracted bones of rats receiving various doses of yeast-fat added to a diet deficient in fat-soluble vitamins.*

| | | | Irradiated fat | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | Specimen I. From incubated yeast: acetone-soluble fraction. Sterol = 9·7 % | | | Specimen II. From yeast direct from brewery. Sterol = 10·2 % | | Specimen III. From incubated yeast hydrolysed Sterol = 2·6 % | Unirradiated fat, from incubated yeast |
| Litter | Sex | Control | | | | | | | |
| Dose of fat in mg. ... | | | 0·005 | 0·025 | 0·125 | 0·025 | 0·125 | 0·125 | 140 |
| Dose of sterol (mg.) ... | | | $\frac{1}{2000}$ | $\frac{1}{400}$ | $\frac{1}{80}$ | $\frac{1}{400}$ | $\frac{1}{80}$ | $\frac{1}{400}$ | — |
| 649 | ♀ | — | — | 60·0 | 57·3 | — | — | — | — |
| 657 | ♂ | 50·0 | — | 55·3 | 57·9* | 53·1 | 56·0 | — | 46·5 |
| 657 | ♀ | 46·8 | — | 57·1 | 56·9 | 53·6 | 56·7 | — | 48·5 |
| 649 | ♂ | 54·6 | 54·9 | — | — | — | — | 55·1 | — |
| 649 | ♂ | 53·0 | — | — | — | — | — | 55·0 | — |
| 648 | ♂ | 53·1 | 55·7 | — | — | — | — | — | — |
| 648 | ♂ | 55·8 | 57·1 | — | — | — | — | — | — |
| 648 | ♀ | 54·7 | — | — | — | 57·1 | 58·9 | 57·7 | — |

B. *Showing total increase in weight (g.) during first three weeks of treatment of the same series of rats with various doses of yeast-fat.*

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 649 | ♀ | — | — | 24 | 41 | — | — | — | — |
| 657 | ♂ | 3 | — | 35 | 46 | 19 | 35 | — | 6 |
| 657 | ♀ | 10 | — | 43 | 56 | 20 | 38 | — | 3 |
| 649 | ♂ | 18 | [illegible] | — | — | — | — | 0 | — |
| 649 | ♂ | 20 | — | — | — | — | — | 22 | — |
| 648 | ♂ | 3 | 23 | — | — | — | — | — | — |
| 648 | ♂ | 12 | 19 | — | — | — | — | — | — |
| 648 | ♀ | 10 | — | — | — | 22 | 25 | 20 | — |

* Value slightly too high, being derived from one heating of the bone only.

*Specimen II.* Doses of 0·025 and 0·125 mg. were given to groups of 3 rats. Some of the rats in these groups were also litter mates of those receiving corresponding doses of Specimen I, and where this is the case, the results are directly comparable. This irradiated fat does not appear to be quite as rich in vitamin D as is Specimen I. The dose of 0·025 mg. shows a considerably higher percentage ash than that yielded by the corresponding controls, but not as high as that yielded by the dose of 0·025 mg. of Specimen I.

*Specimen III.* A dose of 0·125 mg. of this fat was given to 3 rats. In each case the percentage ash is somewhat raised but the results are too fragmentary to draw quantitative conclusions from them. Specimen III was found to have a very low sterol content and there are indications that when irradiated it has less activity than Specimens I and II, whose sterol contents are approximately the same, but of which Specimen I appears to be slightly more active when irradiated. It has already been pointed out [Smedley-MacLean, 1928] that it is not yet known whether both the highly unsaturated sterols in yeast-fat are capable of yielding vitamin D on irradiation or

whether only the ergosterol is so changed. If the latter be the case, the vitamin D content of the fat after irradiation would not bear any constant relation to the total sterol content of the fat.

SUMMARY.

1. Yeast-fat was examined for its content of vitamin A and of vitamin D, the latter both before and after irradiation of the fat.

2. A sample of total fat, derived from pressed brewer's yeast, showed no activity for vitamin A, when a daily dose of 0·18 g. was given to rats on a diet deficient only in vitamin A.

3. A sample of acetone-soluble fat, prepared from yeast which had been incubated in a carbohydrate-phosphate solution, was found neither to promote the growth nor the bone formation of rats on a diet deficient only in fat-soluble vitamins, when a dose of 0·14 g. was given daily. It was therefore concluded that such a sample of yeast-fat was devoid of vitamin D. Luce and Smedley-MacLean had previously found that a sample of total yeast-fat, derived directly from pressed brewer's yeast, did promote growth and calcification of rats. The explanation of the discrepancy probably lies in the different treatment of the yeast from which the specimens of fat were obtained.

4. The same two kinds of yeast-fat, together with a sample of fat prepared from yeast after a preliminary boiling with normal acid, were irradiated with the mercury vapour quartz lamp and fed to rats on a diet deficient in fat-soluble vitamins, in very small daily doses. The best results were given by the fat derived from incubated yeast, which promoted growth and bone formation in a daily dose of 0·005 mg. The quantitative relationship of the vitamin D in the irradiated fat to the two sterols, ergosterol and zymosterol, present in the fat before irradiation is not yet worked out.

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# VI. STRUCTURE AND ENZYME REACTIONS.

## III. THE SYSTEM POLYSACCHARIDE-AMYLASE-PROTEIN.

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In Part II of this series [Przylecki, Niedzwiecka and Majewski, 1927] it was shown that amylase adsorbed upon charcoal has a considerably weaker hydrolytic action than in the absence of the adsorbent; this was explained as being due to the spatial separation of the substrate from the enzyme by fixation on different parts of the adsorbent surface. This model is, however, but a crude imitation of biological conditions, and can serve only as an illustration of the above phenomenon of spatial separation, and as a basis for further research upon the physiology of enzyme reactions.

In this paper only hydrolases of the amylase group will be considered, with the object of establishing that conditions may exist within the living organism which would allow of the spatial delimitation of the enzyme from its substrate. Further, the consequences of the existence of such conditions will be considered.

Lesser explains differences in the kinetics of amylase reactions due to the addition of various substances as being caused by the latter influencing the state or degree of adsorption of the enzyme. In this way he explains the action of adrenaline, and also the influence of alcohol upon the secretion of ptyalin by the submaxillary glands.

As far as the action of adrenaline is concerned, Lesser's [1920] experiments are not entirely satisfactory, since they were carried out upon a perfused liver, in which differences in the permeability of the cell walls might easily take place. Further, other authors have been unable to confirm his results.

The action of alcohol upon the submaxillary glands, causing augmented secretion of amylase, has, on the other hand, been established beyond doubt [Lesser, 1927], but this case is not in any way comparable with conditions such as obtain in the liver, muscles and other organs, in which the ferment is not secreted externally but is used exclusively intracellularly. The conclusions drawn by Lesser from this research cannot, therefore, be applied to organs other than those of external secretion.

No researches have up to the present been undertaken with a view to the elucidation of this problem in organs which metabolise carbohydrates intracellularly, and it is our opinion that this problem should be first investigated upon models before commencing complicated *in vivo* experiments, in which it is difficult to establish the mechanism of the reactions and to be aware of all the factors entering into play. The component parts of these models should be as far as possible identical with those of the living organism.

The first step towards the realisation of these models was to ascertain upon which substances enzyme and substrate could be adsorbed. The spatial delimitation mentioned previously may be greater within the organism as a result of specific differences in adsorption; thus the enzyme and substrate may be adsorbed upon different substances. The possibility of the adsorption of the enzyme is supported by the researches of Hedin [1915], of Starkenstein [1910], who demonstrated the adsorption of amylase by liver components, and of Chrzaszcz and Goralowna [1925] who showed that milk amylase is adsorbed on the fat globules. We have, however, been unable to find any systematic researches on this subject, or upon the similar problem of adsorption of polysaccharides.

It was necessary to find under which conditions and with what facility elution of the adsorbed enzyme and substrate could take place. Further, the degree of activity of the adsorbed enzyme had to be determined and, finally, the kinetics of hydrolysis in the presence of different adsorbents had to be investigated.

All the experiments described in this paper were conducted upon amylase and glycogen or starch. As adsorbents proteins such as egg-white or caseinogen were taken, and minced liver, which is mainly protein, was also used.

## Experimental.

Przylecki's [1918] modification of Pflüger's method for the determination of small quantities (0·2–2·0 mg.) of glycogen or starch was used. A given volume of 1–2 % polysaccharide was shaken for from 2 to 4 hours with a fixed quantity of protein, after which the mixture was filtered. The filtrate was treated with two volumes of 60 % caustic potash, and further treated as for glycogen.

Adsorbed amylase was determined in the following way. Two equal portions of a solution of amylase in 1 % sodium chloride were taken; to one protein was added, and to the other an equal volume of water. Both portions were then shaken for from 2 to 4 hours, the containers being as far as possible completely filled, in order to avoid foaming. Both portions were then filtered, and starch solution was added to equal volumes of the filtrates, which were then placed in a thermostat at 37° for 6–12 hours, when the quantity of reducing sugars formed was determined by Bertrand's method.

For the determination of the velocity of reaction in the presence of

adsorbent, care had to be taken that, before glucose was determined, the protein was completely precipitated. This was done by adding an equal volume of absolute alcohol, and filtering after 4 hours, the residue of protein being then washed three times with 50 cc. of absolute alcohol. The united filtrates were then concentrated to 20–40 cc., *i.e.* until all alcohol had been driven off, made up to a known volume and their glucose content determined by Bertrand's method. In some cases protein was precipitated by Michaelis's method, using colloidal ferric hydroxide; precipitation by means of sodium tungstate and sulphuric acid was also applied. The coagulated egg-white used was in all cases washed for 2 days in running water, in order as far as possible to remove glucoproteins, the prosthetic group of which might interfere with sugar determinations.

As Pringsheim and Winter [1927] have shown that proteins may combine with carbohydrates, an equal portion of protein was, at the end of every experiment, added to those portions to which it had not been added at the beginning, thus minimising this possible source of error.

All experiments were carried out at the same $p_H$.

## Results.

### I. *Adsorption of substrate.*

Glycogen and starch were taken as the substrates, which were adsorbed upon egg-white, caseinogen or liver coagulum (horse or ox). The dispersing medium was either 1 % sodium chloride solution, or the same with the addition of propyl or butyl alcohol.

10 g. of powdered caseinogen adsorbs 20 % of the starch from 50 cc. of a 1 % solution of the latter. Alcohols considerably reduce adsorption, and elute already adsorbed starch, so that in their presence adsorption attains barely 5–7 % (Table I).

Table I. *Adsorption* (%) *of polysaccharides on caseinogen.*

| | | | Adsorption in | | | |
|---|---|---|---|---|---|---|
| | Adsorption | | 0·45 *M* propyl alcohol | | 0·075 *M* butyl alcohol | |
| No. of determinations | Mean | Limits | Mean | Limits | Mean | Limits |
| 10 | 20 | 11·7–32·1 | 6·5 | 4·9–7·4 | 7·1 | 5·1–7·6 |

Table II. *Adsorption* (%) *of polysaccharides on coagulated egg-white.*

| | | | | | Adsorption with | | | |
|---|---|---|---|---|---|---|---|---|
| | No. of determinations | Quantity of egg-white g. | Adsorption | | Propyl alcohol | | Butyl alcohol | |
| Substrate | | | Mean | Limits | Mean | Limits | Mean | Limits |
| Glycogen | 6 | 10 | 31·5 | 27·8–34·3 | 12·9 | 11·8–19·1 | 12·6 | 11·0–19·5 |
| Starch | 10 | 10 | 34·0 | 29·7–38·9 | 14·1 | 12·3–21·6 | 14·7 | 11·8–21·0 |
| „ | 6 | 20 | 55·0 | 49·8–57·1 | — | — | — | — |

10 g. of coagulated egg-white adsorbs from 30 to 40 % of the starch from 50 cc. of its 1 % solution, whilst in the presence of alcohols equilibrium is attained at from 10 to 20 % (Table II). The values obtained using various concentrations of butyl and propyl alcohols (Table III) are of particular importance, since the curves obtained by plotting these values are of the adsorption type, showing that in these phenomena Traube's law is followed. The action of alcohols may therefore be explained as being due to elution, caused by diminution of the adsorbent surface of the protein, which preferentially adsorbs alcohols. Starch and glycogen gave identical results.

Table III. *Elutive action of alcohols.*

| Alcohol | Concentration of alcohol | % eluted starch |
|---|---|---|
| *n*-Propyl | 0·225 % | 41 |
| " | 0·450 % | 59 |
| *n*-Butyl | 0·038 % | 30 |
| " | 0·075 % | 57 |

50 cc. of a suspension containing 10 g. of liver coagulum was mixed with an equal volume of 1 % starch solution. Under these conditions 50–70 % of the starch is adsorbed, so that after 2–4 hours the concentration of the latter falls to 0·25–0·45 % (Table IV). In the presence of alcohols adsorption is greatly diminished, and here, too, alcohols have an intense elutive effect upon adsorbed starch, the concentration of which falls from 0·5–0·7 % to 0·2–0·4 %.

Table IV. *Adsorption* (%) *on liver coagulum.*

| Substrate | No. of determinations | Quantity of coagulum taken g. | In aqueous solution | | With propyl alcohol | | With butyl alcohol | |
|---|---|---|---|---|---|---|---|---|
| | | | Mean | Limits | Mean | Limits | Mean | Limits |
| 2 % glycogen | 6 | 20 | 74·1 | 70·3–80·1 | 31·6 | 23·3–50·8 | 30·9 | 26·1–49·9 |
| 1 % starch | 10 | 10 | 71·3 | 50·3–74·6 | 29·9 | 21·2–40·0 | 28·1 | 20·8–39·4 |

Similar experiments upon glycogen gave identical results. 25 g. of liver coagulum in a volume of 50 cc. adsorbs 70–80 % of a 2 % solution of glycogen; in the presence of alcohols this value falls to 23–50 % (Table IV).

The concentration of free starch, on the addition of quantities of alcohol such as were used by Lesser (0·47 *M* propyl alcohol, 0·075 *M* butyl alcohol) rises by 260 % of that present in solutions containing protein.

II. *Adsorption of enzyme.*

A solution of purified pancreatic amylase was divided into two equal portions. To one was added 20 cc. 1 % sodium chloride solution, and to the other 20 g. of coagulated egg-white. After 2–4 hours, both portions were filtered and 20 cc. of 2 % starch solution were added to equal volumes of the filtrates, which were placed in a thermostat at 37°, and the reducing sugars were then determined. The results, given in Table V, show that 70–95 % of the amylase is adsorbed under these conditions. Alcohols only affect this adsorption to a negligible extent, the maximum elution found being only 5 %.

Table V. *Adsorption (%) of amylase on egg-white.*

| No. of determinations | In aqueous solution | | With propyl alcohol | With butyl alcohol |
|---|---|---|---|---|
| | Mean | Limits | Mean | Mean |
| 8 | 79·0 | 70·5–95·3 | 74·2 | 75·3 |

Liver coagulum was prepared in the following way. Liver was passed through a mincing machine, and for every 100 g. of pulp were added 60–150 cc. of glycerol, 50 cc. of chloroform and 500 cc. of water. The whole was left for 2 days, shaking every 12 hours, after which a further 500 cc. of water were added, and the whole was filtered. The filtrate was placed in a flask plugged with cotton-wool, and left at 37° for 4 days; then for 10 days at room temperature, after which the supernatant fluid had separated from the coagulum which had formed. Amylase was found in both solution and coagulum, the partition being 20–40 % and 60–80 % respectively. Amylase could not be removed from the coagulum by washing, as after prolonged washing the filtrates no longer contained amylase, which was still present in the residue (Table VI) and which remained active even after 10 months. A number of experiments carried out upon the coagulum show that the addition of alcohols leads to only a negligible elution of amylase, barely 3–8 % of which passes into solution.

Table VI. *Amylolytic action.*

Mg. starch decomposed in 24 hours.

| No. of determinations | By ppt. | | By filtrate in aqueous solution | With alcohols | |
|---|---|---|---|---|---|
| | Mean | Limits | Mean | Mean | Limits |
| 5 | 14·1 | 11·9–17·4 | 0 | 0·75 | 0·47–0·92 |

It appears from the above that the passage of a protein from sol to gel may lead to corresponding changes in the dispersion of enzymes present, which, previously uniformly distributed throughout the medium, are now adsorbed upon the protein gel.

Pancreatic amylase was now added to such carbohydrate-free liver coagulum, the whole shaken and filtered after 2–6 hours. Simultaneously the same quantity of amylase as before was added to an equal volume of water, this portion serving as a control. Under such conditions from 50–95 % of the added amylase, according to its concentration, underwent adsorption. This adsorbed amylase was not elutable by alcohols, ether or chloroform to an extent of more than 3–8 % (cf. Table VII).

Table VII. *Percentage of amylase adsorbed upon coagulum.*

| No. of determinations | Aqueous solution | | With butyl alcohol | |
|---|---|---|---|---|
| | Mean | Limits | Mean | Limits |
| 5 | 61·0 | 59·0–75·6 | 58·1 | 54·7–70·2 |

Similarly, glycogen causes a negligible elution of amylase from egg-white or from liver coagulum (Table VIII).

Table VIII. *Elutive action of glycogen.*

| | % adsorption of amylase. | | | |
|---|---|---|---|---|
| | Filtrate without glycogen | | Coagulum + glycogen, filtered | |
| No. of determinations | Mean | Limits | Mean | Limits |
| 3 | 48·1 | 39·9–50·3 | 46·3 | 36·8–49·2 |

III. *Influence of protein upon the velocity of hydrolysis of carbohydrates.*

If the velocities of enzymic hydrolysis of starch in aqueous solution in the presence and absence of coagulated egg-white be compared, it is apparent that the protein retards the velocity of reaction, and experiments made in the presence of buffer solutions show that the observed differences cannot be ascribed to differences in $p_H$.

Table IX shows that the percentage retardation increases with the degree of adsorption. Hence it follows that, in systems containing protein, on which the whole of the amylase present is adsorbed, the velocity of hydrolysis of polysaccharides is a function of the concentration of unadsorbed starch or glycogen, the adsorbed portion of the latter substances taking no part in the reaction.

Table IX. *Influence of coagulated egg-white upon action of amylase.*

| | | Mean values. | | |
|---|---|---|---|---|
| No. of determinations | Quantity of protein g. | % adsorbed starch | Mg. starch hydrolysed | Decrease in hydrolysis in % of that in absence of protein |
| 6 | 0 | 0 | 76·2 | 0 |
| 6 | 10 | 34 | 51·8 | 32 |
| 6 | 20 | 55 | 35·4 | 53 |

If curves be plotted representing velocity of hydrolysis with and without protein, it will be seen that, while in the latter case the velocity of reaction diminishes with time, the curve in the former case is much closer to a straight line. This difference is probably due to the more constant concentration of starch in the presence of the adsorbent, which gives up adsorbed starch to the solution as fast as it is there used up to form glucose. This phenomenon will, however, be more closely investigated in a future research.

Taking into consideration that no elution of enzyme takes place, we see that the addition of alcohols to systems containing coagulated egg-white leads to an acceleration of reaction proportional to the degree of elution of adsorbed polysaccharide. This is well illustrated in Table X from which curves may be constructed representing percentage elution and acceleration of reaction: these curves are very nearly coincident.

Identical results were obtained with liver coagulum and with coagulated egg-white.

Table X. *Action of alcohols.*

% retardation of reaction with respect to that in aqueous solution without protein.

| | Aqueous solution with protein | | Same with alcohols: Propyl alcohol 0·225 *M* | | Propyl alcohol 0·450 *M* | | Butyl alcohol 0·038 *M* | | Butyl alcohol 0·075 *M* | |
|---|---|---|---|---|---|---|---|---|---|---|
| No. of determinations | % retardation | Mg. starch decomposed | % retardation | Mg. starch decomposed | % retardation | Mg. starch decomposed | % retardation | Mg. starch decomposed | % retardation | Mg. starch decomposed |
| 5 | 32 | 51·8 | 18·8 | 61·7 | 13·1 | 66·1 | 22·4 | 59·0 | 13·7 | 65·5 |

If fresh egg-white be taken, the effect is quite different from that of coagulated albumin. Scarcely any retardation of reaction is here observed, and the addition of small quantities of alcohols has no accelerative effect upon the velocity of hydrolysis (Table XI).

Table XI. *Influence of uncoagulated egg-white upon the action of amylase.*

Mg. starch decomposed in 50 cc. reaction solution containing 5 or 10 cc. egg-white solution, 1 % starch and 0·5 % amylase solution, in 0·5 % NaCl. Kept 24 hours at 37°.

| No. of determinations | Without egg-white | | 5 cc. egg-white | | Same with propyl alcohol | | 10 cc. egg-white | | Same with butyl alcohol | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Mean | Limits | Mean | Limits | Mean | Limits | Mean | Limits | Mean | Limits |
| 6 | 68·3 | 62·5–71·9 | 66·8 | 62·2–73·4 | 67·0 | 63·1–69·8 | 65·4 | 61·9–70·6 | 66·1 | 63·7–70·0 |

## Discussion.

The above results show that the velocity of reaction in the system polysaccharide-amylase-protein gel is regulated exclusively by the concentration of unadsorbed substrate. It is a matter of the greatest interest that uncoagulated egg-white present as a sol in the above system does not to any appreciable extent retard the hydrolysis.

It would follow from this that the retardation of reaction depends upon the state of dispersion of the adsorbed enzyme. This may be due either to the non-adsorption by protein sols of the enzyme or substrate or of both, or it may be that adsorption takes place, but does not always retard reaction. In order that reaction should be retarded, the number of collisions between enzyme and substrate must be diminished. An active adsorbed enzyme may combine with the substrate as easily as when unadsorbed: in solution, therefore, in spite of both or either of the constituents, *i.e.* enzyme and substrate, being adsorbed, the number of collisions taking place in a given time need not necessarily be diminished. Such a diminution could take place if the state of the adsorbent changes from that of a sol to that of a suspension. Passage from the liquid to the solid phase and changes in the size of the particles of the adsorbent may also influence the reaction.

This problem is now being investigated in our laboratory.

Adsorption of amylase upon protein does not affect its activity, while the action of alcohols is confined almost exclusively to elution of the polysaccharide, that of the enzyme being negligible.

These results are identical with those obtained using charcoal as the adsorbent (Part II).

Alcohols produce elution of polysaccharides, and are, in all probability themselves strongly adsorbed, this adsorption following Traube's law. It is therefore extremely probable that the accumulation of lipin-soluble narcotics in the cells is due not only to their collection on lipin surfaces but also to their adsorption upon protein surfaces.

From the above data, obtained exclusively from biological material, similar to that of which cells are composed, the following conclusions may be drawn. Starch and glycogen, present normally in cells in small quantities, are mainly adsorbed upon proteins, which are present to the extent of 10 % in the cell fluid. The concentration of these polysaccharides in the solvent itself is probably less than 20 % of their total amount. This estimate of 20 % may in all probability be still further reduced, if the presence of colloidal adsorbents other than proteins be taken into consideration.

Whilst this view is not universally accepted, yet it would appear that the concentration of unadsorbed amylase in the cell must be almost negligible, as is shown by Lesser's observations as well as by our observation that, where concentrations of amylase and of protein comparable to those of the cell are taken, over 90 % of the enzyme undergoes adsorption.

Under such conditions, changes in the velocity of hydrolysis of polysaccharides may be ascribed to elution of substrate, of enzyme, or of both, to the release of the enzyme or substrate from some part of the cell where it is not free to enter into reaction, or to changes in the state of dispersion of the protein. The last mentioned factor, without any changes in the degree of adsorption of the constituents, may, *in vivo*, appreciably affect the kinetics of the reaction.

It should also be borne in mind that all the above factors may exert their influence simultaneously, and that, in all attempts at the explanation of the reasons for differences in the velocity of the biological hydrolysis of polysaccharides they must be taken into consideration, as well as other factors which catalyse reaction in homogeneous systems, such as hydrogen and other ions.

Further, if the reversibility of the reaction glycogen → glucose be accepted, the degree of hydration of the sphere of action of the enzyme must be an important factor in the reaction. It is very probable that the degree of hydration of the absorbent decides the direction of enzymic reaction; should this be the case, this would be a further factor influencing the velocity of hydrolysis without affecting the degree of adsorption. In this connection, it is of interest that, according to Chick [1914], 1 g. of protein may bind as much as 2·1 g. of water, and it is conceivable that, within the cell, salts or other substances may affect this degree of hydration.

The acceleration of the velocity of hydrolysis caused by the addition of narcotics such as chloroform, ether or alcohols might on the basis of this

research be explained as being due exclusively to elution of polysaccharide. While such an explanation appears to fit in very well with experimental findings, it should not be forgotten that in our case we are dealing with a system simpler than that present within the cell, as it contains only one of the components of the latter, namely, protein. The possibility remains that within the cell the enzyme is adsorbed not only on its protein elements, but also upon lipin or nucleoprotein surfaces, from which elution by narcotics may be much greater than from proteins.

Dauwe [1905] has shown that pepsin, rennin and $\beta$-glucosidase may be adsorbed upon cholesterol and lecithin (see also Chrzaszcz). This problem may, therefore, be completely elucidated only after all possible enzyme adsorbents present in the cell have been investigated.

There can, however, be no doubt that elution of substrate from protein, or change in the state of dispersion of the latter, plays an important part in the acceleration of the velocity of enzymic hydrolysis of polysaccharides due to the addition of narcotics.

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# VII. THE CAUSE OF ANDREWES'S DIAZO-TEST FOR RENAL INEFFICIENCY.

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Andrewes [1924] noted a peculiar colour reaction in uraemic sera when performing Van den Bergh's diazo reaction. Hewitt [1925] simplified the test, and as a result of his investigations suggested tentatively that a cyclic amine, such as histamine or tyramine, might be responsible. A summary of previous work, including a description and the clinical value of the test, has been reported elsewhere [Harrison and Hewitt, 1927]. In the present paper the steps leading to the identification of the substance responsible for the reaction will be given.

### *Hewitt's hypothesis not confirmed.*

If Hewitt's suggestion were correct, a suitable extract of uraemic plasma giving a positive Andrewes's reaction would probably give Pauly's diazo reaction. In other words, Andrewes's reaction might be merely another way of performing the Pauly test. The latter, however, is inhibited by alcohol, whereas in the former about 50 % of alcohol is present. The following table shows that neither histamine nor tyramine gives Andrewes's reaction, and conversely that extracts of positive sera do not give Pauly's reaction.

| Substance tested | Andrewes reaction | Pauly reaction |
|---|---|---|
| Alcoholic extracts of uraemic sera... ... ... | Strongly positive | Negative |
| Same, but alcohol removed ... ... ... | Positive | „ |
| Histamine (ergamine phosphate), aqueous 0·01 % | Negative | Positive |
| Same, with alcohol to 50 % ... ... ... | „ | Negative |
| Tyramine (acid phosphate), aqueous 0·1 % and greater dilutions ... ... ... ... ... | „ | Positive |
| Same, with alcohol ... ... ... ... ... | „ | Negative |
| Tyrosine, 0·1 to 0·01 % ... ... ... ... | „ | Positive |
| Histidine, 0·1 to 0·01 % ... ... ... ... | „ | „ |
| Tryptophan ... ... ... ... ... ... | „ | — |
| Indole... ... ... ... ... ... ... | „ | — |
| Skatole ... ... ... ... ... ... | „ | — |

### Investigation of positive sera.

It was found that *X*, the substance responsible for the reaction, was very sensitive to changes in the hydrogen ion concentration. It was apparently decomposed by heating in either acid or ammoniacal solution. It could safely

be heated to boiling in neutral organic solvents. It could be boiled in neutral aqueous solution without decomposition, and such a solution could be evaporated to dryness. *X*, in a grossly impure state, was soluble in water (very); aqueous hydrochloric acid (slowly decomposed); ethyl alcohol (slightly); amyl alcohol (slightly) whether the reaction was slightly acid, neutral, or alkaline; acetone (fairly); butyl alcohol (slightly), and aqueous pyridine. It was insoluble in ether, chloroform, and light petroleum.

The fact that it was possible to *boil* the diazo reagent with an alcoholic extract of serum was puzzling because most diazo reactions must be carried out in the cold. The presence of alcohol was not essential. After removing it by evaporation, the residue, dissolved in water, also gave the test.

It was possible to concentrate the colour reaction some 20 to 50 times, but all attempts to make even a rough separation of *X* failed.

*X in normal urine.*

Hewitt [1925] noted that alcoholic extracts of some normal urines gave a reaction very like that of Andrewes. One of us (G.A.H.) remembered that Hewitt's positive observations were mostly obtained from a subject whose urine was often used in class-work for demonstrating a positive indican reaction. This suggested that *X* might be indican.

Potassium indoxyl sulphate reacts with phenyldiazonium chloride, and on making alkaline, the colour change in dilute solution is very similar to that in Andrewes's reaction [see Neubauer-Huppert, 1913]. Moreover, the methods we had found the best for concentrating the colour reaction both in uraemic sera and in normal urines, were very like that recommended by Hoppe-Seyler [1882] for separating indican from urine. Again, indicanaemia is well known to occur in uraemia, and a marked excess of indican in the blood is found in no other condition. We found that all our sera giving a positive Andrewes's reaction also gave a positive Jaffé test for indican, the latter reaction was, however, less sensitive than the former. If *X* were indican it might account for the observation that an alcoholic extract of a positive serum had either to stand for many hours at room temperature, or to be boiled with the diazo reagent. Potassium indoxyl sulphate is hydrolysed by dilute acid slowly at room temperature, but rapidly when boiled, to liberate free indoxyl which then reacts with the diazo reagent.

An attempt was therefore made to isolate indican in a state of purity from urine by the methods of Baumann and Brieger [1879] and of Hoppe-Seyler [1882], and by modifications of these. A very small yield of indican was obtained from horse's urine[1], and from the combined urines of human patients showing marked indicanuria, but the salt was far from pure. It was noted, however, that Andrewes's reaction and Jaffé's test for indican ran

[1] We are greatly indebted to Dr R. A. O'Brien, Director of the Wellcome Research Laboratories, for the supply of horse's urine. Horse's urine is 10 to 25 times as rich in indican as normal human urine.

parallel. *X* was therefore either indican or some substance concentrated along with indican at each stage of the process.

It therefore became necessary to obtain pure indican in some other way and the method of Jolles and Schwenk [1915], with slight modifications[1], was found to be feasible. *N*-Acetylindoxyl and potassium indoxyl sulphate both gave a typical Andrewes's reaction, in either alcoholic or aqueous solution. Moreover, the reactions were positive when solutions of each of these substances in normal human sera were tested, the solutions containing 1 mg. per 100 cc. serum or being even more dilute. This is important because the reported findings for indicanaemia in uraemia [see Wells, 1925] range from 0·2 to 2·2 mg. per 100 cc. serum.

## Conclusion.

It is therefore concluded that the substance in uraemic sera responsible for Andrewes's reaction is an indoxyl compound, presumably potassium indoxyl sulphate (indican), or possibly in part indoxyl glycuronate.

We are greatly indebted to several friends, and to Dr W. H. Hurtley in particular, for advice and helpful criticism. We wish to thank Messrs Bayer Products Ltd. for their generous gift of *N*-acetylindoxyl.

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[1] The preparation from *N*-acetylindoxyl, and the isolation of indican from urine will be reported later.

# VIII. THE CHLORIDES AND INORGANIC CONSTITUENTS OF THE SERUM AND CEREBROSPINAL FLUID IN MENINGITIS.

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## Introduction.

It has been established for many years that in meningitis and especially in tuberculous meningitis the concentration of chloride in the cerebrospinal fluid falls, and this fact has become of considerable value in diagnosis. Mestrezat [1912] was one of the first to note that the chloride concentration of the cerebrospinal fluid was higher than that of the serum, and he further reported its decrease in meningitis.

In health the concentration of chloride in serum is 0·58 % (100 millemoles per litre) and in the cerebrospinal fluid 0·73 % (125 *mM.*). Attempts have been made to explain this difference on physical grounds, by the difference in water content and protein concentration of the two fluids leading to an unequal distribution of ions in accordance with the Donnan law [Hamilton, 1925]. Mestrezat and Lebebt [1921] produced by dialysis *in vitro* and *in vivo* fluids very closely resembling cerebrospinal fluid, and showing a higher chloride content than the serum from which they were formed. Duke-Elder [1927] found a higher chloride content in the aqueous humour than in the corresponding serum; he attributed this to the Donnan effect and found no evidence that the aqueous humour is a secretion. The aqueous humour is very closely related to the cerebrospinal fluid. Fremont-Smith [1927] reviews the evidence for and against the secretion of the cerebrospinal fluid by an essentially vital activity of the choroid, and accepts it as proven that its composition can be explained by the laws of filtration and dialysis.

Against accepting this conclusion too hurriedly it may be observed that the value of the ratio $Cl_{serum}/Cl_{C.S.F.}$ is always much smaller than the value of R predicted from the formulae of Van Slyke, Wu and McLean [1923], and much less than the ratio for Cl actually found by Hastings, Salvesen, Sendroy and Van Slyke [1927] in a study of the relation of oedema fluid to serum. And further according to Donnan's law

$$R = \frac{Cl_{serum}}{Cl_{C.S.F.}} = \frac{HCO_{3\,serum}}{HCO_{3\,C.S.F.}} = \frac{A_{serum}}{A_{C.S.F.}} = \frac{B_{C.S.F.}}{B_{serum}} = \frac{H_{C.S.F.}}{H_{serum}}$$

where A = total univalent anions and B = total univalent cations. In the

study of oedema fluid just referred to there was a much closer approximation to the above conditions than has yet been demonstrated for cerebrospinal fluid, but it might be argued that the great stagnation of oedema fluid would allow more complete equilibration to take place. There is yet much to learn as to the properties of the separating membranes of the body. Since the ratio for bicarbonate departs entirely from that predicted, the membranes of the choroid and meninges would appear to be impermeable to this ion, as the membrane of the red blood cell is impermeable to basic ions [Van Slyke, Wu and McLean, 1923].

Recently Cohen [1923, 1927] has attempted to explain the fall in the chlorides of the cerebrospinal fluid in meningitis by attributing it to an increase in permeability of the choroid plexuses so that the chloride concentration approaches more closely to that of the serum. In favour of this hypothesis he brings evidence to show that in meningitis the magnesium and inorganic phosphate concentration of the fluid approach that of the serum; that fibrinogen, bile pigments and "complement" appear in the cerebrospinal fluid; and that certain foreign substances such as nitrates and iodides, which do not normally pass into the cerebrospinal fluid from the blood stream, will then do so.

But the decrease of chlorides in tuberculous meningitis from 0·72 % to as low as 0·55 % is unassociated with a great increase of protein in the cerebrospinal fluid, and therefore such a great fall would not be required to bring the chlorides into equilibrium with the serum if the serum chlorides remained unchanged.

It is the object of this preliminary paper to show that the fall of chlorides in meningitis is due to other causes than increased permeability of the meninges.

## Experimental.

Four patients have been studied; two had tuberculous meningitis, one meningococcal meningitis and one streptococcal meningitis. All were proven bacteriologically. Cerebrospinal fluid was withdrawn by cisternal or lumbar puncture into a paraffined syringe without contact with air; 15 to 20 cc. were taken. Immediately afterwards arterial blood was drawn from the femoral artery with similar precautions and the serum was separated as soon as possible.

Chloride was determined by Van Slyke's method [1923], $CO_2$ by the constant volume apparatus of Van Slyke and Neill [1924], total fixed base by the benzidine method of Stadie and Ross [1925], inorganic phosphate by the method of Benedict and Theis [1924], and $p_H$ by the Hastings and Sendroy [1924] colorimetric method. The $p_H$ and $CO_2$ analyses on the cerebrospinal fluid were made without delay. The bicarbonate was calculated from the Hasselbalch equation taking 6·10 as the $p_K$ of serum and 6·13 as that of cerebrospinal fluid [Shohl and Karelitz, 1926]. The "undetermined acids" were

determined by subtracting from the total base the sum of the base bound by the chloride, bicarbonate, phosphate and protein; the base bound by the protein was derived from the formula $(BP)_s = 0{\cdot}097\ (P)_s\ (p_{Hs} - 5{\cdot}26)$ [Hastings, *et al.*, 1927].

The results are shown in Table I together with the mean of a series of 26 probably normal cerebrospinal fluid and serum analyses published by Hamilton [1925]. He assumed that 1 litre of serum contained 930 g. of water and that 1 litre of cerebrospinal fluid contained 1 kg. of water. This figure for serum agrees with the one we obtained by calculation from the protein content in Cases I and II so we have adopted it throughout, and the results are given as millemoles per litre of water.

Table I.

| Case | Diagnosis | Chloride: Serum water mM per litre | Chloride: C.S.F. water mM per litre | $\frac{Cl_{serum\ water}}{Cl_{C.S.F.\ water}}$ | Bicarbonate: Serum water mM per litre | Bicarbonate: C.S.F. water mM per litre | $\frac{B\ HCO_{3\,serum\ water}}{B\ HCO_{3\,C.S.F.\ water}}$ | Total base: Serum water mM per litre | Total base: C.S.F. water mM per litre | $\frac{B_{C.S.F.\ water}}{B_{serum\ water}}$ |
|---|---|---|---|---|---|---|---|---|---|---|
| | Normal | 111 | 124 | 0·895 | 25 | 21 | 1·19 | 164 | 156 | 0·95 |
| I | Tuberculous meningitis | 90 | 105 | 0·86 | 47 | 32 | 1·47 | 172 | 151 | 0·88 |
| II | Tuberculous meningitis | 97<br>91 | 108<br>102 | 0·90<br>0·89 | 33<br>31 | 28<br>25 | 1·18<br>1·24 | 172<br>162 | 144<br>138 | 0·84<br>0·85 |
| III | Meningococcal meningitis | 77 | 89 | 0·87 | 44 | 40·5 | 1·09 | — | 144 | |
| IV | Streptococcal meningitis | 86 | 96 | 0·90 | 24 | 14 | 1·71 | 146 | 124 | 0·85 |

| Case | Diagnosis | Inorganic phosphorus: Serum water mM per litre | Inorganic phosphorus: C.S.F. water mM per litre | Other acids: Serum water mM per litre | Other acids: C.S.F. water mM per litre | $p_H$: Serum | $p_H$: C.S.F. | Protein: Serum % | Protein: C.S.F. % | Remarks |
|---|---|---|---|---|---|---|---|---|---|---|
| | Normal | 1·2 | 0·6 | 10 | 10 | — | — | — | — | Mean of 26 analyses of epileptics |
| I | Tuberculous meningitis | 1·8 | 1·0 | 15 | 12 | 7·60 | 7·42 | 6·5 | 0·09 | 13. iii. 27 |
| II | Tuberculous meningitis | 1·4<br>1·4 | 0·8<br>0·9 | 26<br>24 | 7<br>9 | 7·48<br>7·48 | 7·36<br>7·31 | 6·2<br>6·3 | 0·14<br>0·14 | 6. x. 27<br>18. x. 27. Death on 24. x. 27 |
| III | Meningococcal meningitis | — | — | — | 14 | 7·48 | 7·40 | — | 0·4 | 6. iv. 27. Venous serum |
| IV | Streptococcal meningitis | — | — | 20 | 13 | 7·20 | 7·0 | — | — | Death next day |

*Chloride.* In all the cerebrospinal fluids a marked decrease in chloride concentration had occurred; the lowest was found in the case of chronic meningococcal meningitis in which it was 0·52 %. On examination of the figures for serum chloride it was at once apparent that there had been a corresponding decrease in these. It was found that the ratio $Cl_S/Cl_{C.S.F.}$ was remarkably constant and agreed well with that calculated from Hamilton's series. The constancy of this ratio excludes the possibility that a change of permeability was the cause of the fall of cerebrospinal fluid chlorides, and is an additional fact supporting the view that the Donnan law has an important rôle in determining the composition of the fluid.

Since the serum and cerebrospinal fluid remain almost neutral in reaction, such a decrease in chlorides must be accompanied by an increase in the other anions, bicarbonate, phosphate, protein or other acids, or by a decrease in the cations. The changes in bicarbonate, total base and "undetermined acids" were therefore studied.

*Bicarbonate and* $p_H$. In Cases I and II there was a great increase in bicarbonate, but in Case I a small amount of alkali had been given by mouth during the week preceding our observations; both showed a high $p_H$ in the serum with lower figures for the cerebrospinal fluid. In Case II the bicarbonate was moderately high; the reaction of the serum was normal and the cerebrospinal fluid was slightly more acid. In Case IV there was a marked fall in serum $p_H$ with normal bicarbonate, a state of "uncompensated $CO_2$ excess," possibly due to insensitiveness of the respiratory centre. The cerebrospinal fluid showed a great fall of bicarbonate and an extremely low $p_H$. That the cerebrospinal fluids were regularly so much more acid than the sera was attributed to the presence of infection within the meninges.

*Total base.* The total base in the cerebrospinal fluid was rather less than was found in Hamilton's cases, and this was more marked in Case IV in which the serum base was also definitely reduced. The ratio $B_{C.S.F.}/B_S$ was fairly constant in our cases but considerably lower than the ratio found by Hamilton; they agreed better with, but are lower than, the corresponding values for the chloride ratio. The amount of bivalent base is so small that we have neglected it.

*Undetermined acids.* There was much less undetermined acid in the cerebrospinal fluid than in the serum and no significant change took place. The increase of lactic acid in meningitis reported by Glaser [1926] to about 10 millemoles per litre of cerebrospinal fluid was not suggested by our figures.

## Summary.

1. It seems evident that the fall of chloride concentration in the cerebrospinal fluid in meningitis is dependent on a similar fall in the chloride concentration of the serum. The relative concentrations in cerebrospinal fluid and serum remain the same.

2. The associated disturbances are an increase, sometimes large, of the bicarbonate in both fluids and a small decrease in total base in the cerebrospinal fluid. Sometimes a greater depletion of base occurs in cerebrospinal fluid and in serum, and in such cases the bicarbonate does not rise and may be low.

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# IX. THE BIOLOGICAL SIGNIFICANCE OF THE UNSAPONIFIABLE MATTER OF OILS.

## III. FISH-LIVER OILS.

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In a previous paper [Channon, 1926] it was reported that when the unsaturated hydrocarbon squalene was administered to rats, partial absorption of the hydrocarbon occurred. At the same time, there was a considerable increase in the amount of the unsaponifiable fraction of the liver, and the presence of squalene could be demonstrated in that fraction. The increase in the amount of the unsaponifiable matter of the liver was chiefly caused by a rise in the amount of cholesterol to more than twice its normal value. This marked rise in liver cholesterol suggested that squalene or some substance of similar chemical nature might be the precursor of cholesterol in the animal body. On the other hand, the increase in the amount of the unsaponifiable fraction was not entirely accounted for by the amount of squalene present and the increase in cholesterol. It thus appeared possible that the increase in sterol was the result of some process existing for the purpose of keeping the ratio of the amounts of cholesterol to that of the other unsaponifiable substances present as constant as possible. It was with a view to obtaining further information on these points that the work described in this paper was undertaken.

It seemed probable that a study of the amount of sterol present in the unsaponifiable fractions of a number of liver oils might throw some light on the question of a possible relationship existing between the amounts of sterol and those of the other unsaponifiable substances present. Fish-liver oils were chosen for this purpose for a number of reasons. Firstly, there is present in a number of these oils a high percentage of unsaponifiable matter and the study of these should make any such relationship the more apparent. Secondly, it was desired at the same time to obtain information regarding the distribution of squalene, for although the amount of the hydrocarbon present in a large number of sharks of Japanese waters has been investigated by Tsujimoto and Toyama [Tsujimoto, 1916, 1918, 1920, 1; Toyama, 1922, 1923, 1] no observations as to its presence in the commoner fish of British waters are available. Further, evidence as to the possibility of squalene being the precursor of cholesterol in the animal body might be forthcoming as the result of a study of the amount of sterol in the squalene-containing oils.

Another question on which information has been sought is the source from which squalene is derived. Whether certain fish are able to synthesise squalene or whether the hydrocarbon is derived from the food is a question which cannot be readily answered. A similar type of experiment to that reported on cholesterol synthesis in the rat [Channon, 1925] suggests itself, but unfortunately the fact that the squalene-containing fish available in home waters are viviparous renders such an experiment almost impracticable. It was desirable, therefore, in addition to determining whether the hydrocarbon is present in fish-liver oils generally, to prepare the unsaponifiable fraction of a number of samples of plankton in order to determine whether this material, which is the ultimate food of fish, contained it.

With these problems in view, the unsaponifiable matter has been prepared from a number of fish-livers and fish-liver oils: the sterol contents of these materials have been determined, and they have been examined for the presence of squalene: similar observations have been made on a number of samples of plankton and a comparison has been drawn with the unsaponifiable fraction of certain mammalian livers.

## Experimental.

The livers of the fish used in this work were obtained by the author from the Marine Biological Station, Plymouth. The livers of the fish caught in shallower waters were removed immediately from the fish as soon as landed by the trawler belonging to the Station. Those of the fish caught in deeper waters were procured in the fish market from the fish freshly landed from the deep sea trawlers. As the latter are at sea for varying periods, the livers of these fish were seldom fresh.

In attempting to discover whether squalene was present in the livers used, it was clearly desirable first to prepare the unsaponifiable fraction of these oils because, since the proportion of unsaponifiable matter in the majority of them is very low, the attempt to find squalene in the original oil might fail on account of the small quantities present. Accordingly, the livers of the fish after weighing were covered with 5 % aqueous potassium hydroxide and stored in well-stoppered bottles until opportunity arose for continuing the extraction, which in most cases was several weeks after the material had been brought to London.

### *Preparation of the unsaponifiable matter.*

The methods used for the preparation of the unsaponifiable matter were those already described by the author [Channon, 1925]. Briefly, after complete solution of the livers in the hot aqueous 5 % potassium hydroxide and considerable dilution of the resulting fluid with water, an exhaustive ether extraction was made. After washing with water, the ether extract was evaporated to dryness, and the resulting oil dissolved in hot alcohol and re-saponified with sodium ethoxide. The fluid obtained by pouring the solution of the re-saponified product into water was again exhaustively extracted with ether,

the ether extract was washed with water and evaporated to dryness *in vacuo*. In this way there was obtained the unsaponifiable fraction. In a number of cases where large amounts of liver were used, it was necessary to carry out a third saponification on account of the high content of oil of some of the livers. It is clear that the extraction of an aqueous digest of liver tissue in bulk cannot be strictly quantitative. There are troubles due to emulsions which are not easily overcome. Hence, although up to twenty ether extractions were made in some cases, it is not claimed that the yields obtained were always the maximum possible. There is probably in these cases a loss of not more than 5 %. This is not considered of importance here, as the work was designed to obtain figures showing what livers, if any, contained a large amount of unsaponifiable matter, and losses of this order may be neglected. It may be taken also that the losses were general and not losses of the less soluble constituents, so that the percentages of sterol present may be regarded as sufficiently accurate for the purposes planned.

A number of the oils used were kindly supplied by Prof. I. M. Heilbron of Liverpool University. These were saponified twice, first with alcoholic potassium hydroxide and then with sodium ethoxide. The amount of sterol present in the unsaponifiable fractions was determined on aliquot portions by the digitonin method of Windaus [1910]. The possible presence of squalene was sought by attempting to prepare the characteristic crystalline hexahydrochloride $C_{30}H_{56}Cl_6$ in the following manner. The solution of unsaponifiable matter was evaporated to dryness *in vacuo* and then dissolved in the minimum amount of boiling water-free acetone. The solution was allowed to cool and then filtered rapidly. In this way, a large proportion of the sterol present was removed and the other substances present were obtained in strong solution in acetone. These cooled acetone solutions were then saturated with dry hydrochloric acid gas. After cooling on ice, any precipitate was filtered off, washed with ether and recrystallised from acetone. Its characteristic crystalline form and melting point were used for the identification of the hydrochloride. Acetone was used as a solvent for the preparation of the hydrochloride in preference to the solvents previously used [Channon, 1926; Drummond, Channon and Coward, 1925] on account of the higher yields so obtained [Heilbron, Kamm and Owens, 1926]. Exactly similar methods were used for the preparation and subsequent treatment of the unsaponifiable fraction from the plankton. In those cases where the percentage of the unsaponifiable fraction was high its iodine value was determined.

## Results.

The weights of liver or liver oil used and the yield of the unsaponifiable fractions, together with the sterol calculated as a percentage of the unsaponifiable fraction, are given in Table I.

By the kindness of the Director of the Marine Biological Station, Plymouth, a number of samples of plankton were placed at the author's disposal. The

## Table I.

| Order SELACHII<br>Sub-order | Family | | | Liver g. | Liver oil g. | Unsap. matter g. | Unsap. matter as % of liver or oil | Sterol as % of unsap. matter | Iodine value |
|---|---|---|---|---|---|---|---|---|---|
| Notidani | Notidanidae | *Notidanus griseus* | six-gilled shark | — | 8·03 | 1·1184 | 13·92 | 44·8 | 72·4 |
| Scylloidei | Scyliidae | *Scyllium canicula* | spotted dogfish | 410 | — | 1·3432 | 0·328 | 64·0 | — |
| | | | | — | 6·63 | 0·1270 | 1·915 | 44·7 | — |
| | Lamnidae | *Lamna cornubica* | porbeagle | — | 10·22 | 0·1150 | 1·126 | 40·9 | — |
| | Charchariidae | *Mustelus vulgaris* | smooth hound | 200 | — | 1·0010 | 0·500 | 39·3 | — |
| | | *Galeus vulgaris* | tope | — | 7·76 | 0·2968 | 3·824 | 20·46 | — |
| Squaliformes | Squalidae | *Spinax niger* | spinax | — | 8·55 | 5·7387 | 67·13 | 0·61 | 343·0 |
| | | *Acanthias vulgaris* | picked dogfish | — | 7·75 | 0·9675 | 12·48 | 7·96 | 67·4 |
| | | *Scymnodon ringens* | | — | 8·35 | 2·7969 | 33·50 | 1·47 | 73·7 |
| | | *Scymnorhinus lichia* | darkie Charlie | — | 8·90 | 7·2541 | 81·49 | 0·13 | 341·5 |
| | | *Lepidorhinus squamosus* | | — | 8·72 | 6·3539 | 72·88 | 0·53 | 362·7 |
| Raiiformes | Squatinidae | *Rhina squatina* | | 815 | — | 4·6420 | 0·57 | 72·6 | — |
| | Torpedinidae | *Torpedo nobiliana* | electric ray | — | 8·94 | 1·4867 | 16·63 | 10·59 | 79·5 |
| | Raiidae | *Raia batis* | skate | — | 10·48 | 0·2210 | 2·11 | 51·45 | — |
| | | ,, *circularis* | sandy ray | 500 | — | 0·9288 | 0·185 | 64·5 | — |
| | | ,, *clavata* | thornback ray | 2500 | — | 8·535 | 0·341 | 38·2 | — |
| | | | | — | 9·22 | 0·2287 | 2·48 | 24·66 | — |
| | | ,, *sp.* | black skate | 1130 | — | 9·060 | 0·802 | 64·7 | — |
| Order TELEOSTEI | | | | | | | | | |
| Anacanthini | Gadidae | *Gadus luscus* | bib | 45 | — | 0·1568 | 0·348 | 46·4 | — |
| | | ,, *merlangus* | whiting | 27 | — | 0·1198 | 0·444 | 42·9 | — |
| | | ,, *minutus* | poor cod | 185 | — | 0·8192 | 0·443 | 81·4 | — |
| | | ,, *pollachius* | pollack | 80 | — | 0·3560 | 0·445 | 65·9 | — |
| | | ,, *virens* | coalfish | 930 | — | 2·0793 | 0·224 | 59·4 | — |
| | | *Merluccius vulgaris* | hake | — | 10·34 | 0·1443 | 1·397 | 56·4 | — |
| | | *Phycis blennoides* | greater forkbeard | — | 7·20 | 0·1702 | 2·363 | 51·4 | — |
| Apodes | Anguillidae | *Conger vulgaris* | conger | 745 | — | 2·5868 | 0·347 | 58·2 | — |
| Acanthopterygii | Triglidae | *Trigla cuculus* | red gurnard | 165 | — | 1·2082 | 0·731 | 83·5 | — |
| | | ,, *gurnardus* | grey gurnard | 65 | — | 0·7358 | 1·132 | 63·9 | — |
| | | ,, *lineata* | streaked gurnard | — | — | 0·0507 | — | 73·2 | — |
| | Pleuronectidae | *Pleuronectes platessa* | plaice | 91 | — | 0·4138 | 0·455 | 74·9 | — |
| | | ,, *microcephalus* | lemon sole | 65 | — | 0·0664 | 0·102 | 54·2 | — |
| | Callionymidae | *Callionymus lyra* | dragonet | 80 | — | 1·3462 | 1·683 | 69·3 | — |
| Pediculati | | *Lophius piscatorius* | angler | 420 | — | 2·6686 | 0·636 | 68·9 | — |

unsaponifiable fractions from these and also from a further sample obtained from the Scottish Marine Biological Station, Keppel Pier, Millport, Bute, were prepared. The percentage of sterol precipitated by digitonin and the iodine values of the sterol-free fractions are given in Table II.

Table II.

| Source and nature of plankton | | Wt. of unsap. fraction g. | Sterol as % of unsap. fraction | Iodine value of sterol-free material |
|---|---|---|---|---|
| Millport. | Large animal | 4·57 | 0·45 | 50·5 |
| Plymouth. | Large animal | 0·1384 | 29·48 | 106·3 |
| | Medium animal | 0·0804 | 12·63 | 77·8 |
| | Small animal and some plants | 0·5436 | 21·20 | 75·1 |
| | Mostly plant life: diatoms | 0·1206 | 7·49 | 90·2 |

Squalene was not found in any of these fractions. One point of interest regarding the unsaponifiable fraction of the specimen obtained from Millport is that, although carefully prepared in a current of nitrogen, it failed to give the vitamin A reaction with arsenious chloride [Rosenheim and Drummond, 1925].

## Discussion.

### 1. *The distribution of squalene.*

Squalene was not found in any of the unsaponifiable fractions examined save in those from the livers of three members of the Squalidae family—*Spinax niger*, *Scymnorhinus lichia* and *Lepidorhinus squamosus*, in which the percentages of unsaponifiable matter were 67·13, 81·49 and 72·88 respectively. This limited distribution of the hydrocarbon makes it of interest to compare the results of Tsujimoto [1920, 1], who found it present in the livers of 16 of the 36 species of the Elasmobranchs of Japanese waters which he examined. Only in liver oils having a specific gravity of less than 0·9 at 15° was squalene present in quantity; in three oils of a slightly greater specific gravity, it was present in small amounts only. It is present chiefly in the liver oils of the family Squalidae, but is also present in certain members of the Cetorhinidae, Chlamydoselachidae, Dalatiidae and Scylliorhinidae. The percentage of unsaponifiable matter in the oils of specific gravity of less than 0·9 may vary from 37 to 90·2 %, with iodine values from 130 to 345; in those of higher specific gravity, the percentage of unsaponifiable matter varies from 0·9 to 23·2 % and the iodine values from 91 to 197. From these results it is seen that squalene is found chiefly in the Squalidae, although curiously enough not every member of that family contains it. This curious distribution of the hydrocarbon is perplexing. It is unlikely that squalene is derived from the food for, if such were the case, it seems probable that it would be present in the livers of fish generally, and yet not only is it limited in its distribution, but it is absent from those samples of plankton examined. Further, the fact

that the feeding habits of all the members of the Squalidae family appear similar is also evidence against it being a product derived from the food. Nor does it seem reasonable to suppose that a fish should possess the power of replacing the greater part of its liver oil by a hydrocarbon derived from the food. On the other hand, if the hydrocarbon is synthesised by certain fish, it is also difficult to understand its distribution or to account for its presence in very large amounts in the livers of certain of the Squalidae and its absence from others.

Another point worthy of notice is that squalene also occurs in the egg oils of *Chlamydoselachus anguineus* and *Lepidorhinus kinbei* [Tsujimoto, 1920, 2]. Further, Heilbron, Kamm and Owens [1926] reported that they examined 115 female *Etmopterus spinax*. In some of these eggs in a comparatively undeveloped condition were found and all contained squalene. In others, where development had proceeded far, the yolk sac was devoid of squalene, showing that absorption of the substance had occurred during development.

The absence of squalene from the Teleostei is interesting, for it will be remembered that it was reported to be present in very small amounts in cod-liver oil [Drummond, Channon and Coward, 1925], and Weidemann [1926] confirms the presence of the hydrocarbon in that oil. It is possible, however, that the hydrocarbon is not present in cod-liver oil, other than fortuitously, for in the degutting of the cod at sea, it is usual for the livers of fish other than cod to be mixed with the cod-livers themselves.

2. *The yield of unsaponifiable matter.*

The figures presented show that there is a large variation in the amount of unsaponifiable matter yielded by the livers of different fish. Strict comparison between the Selachii and Teleostei is not possible, as in the former the liver oils were used in most cases and in the latter the livers themselves. Certain facts stand out, however. It is only among the Selachii that there are found fish with a high content of unsaponifiable matter in their livers. Thus, among the Squalidae, in the three fish in which squalene is present, the values for the percentages of unsaponifiable matter are 67·13, 81·49 and 72·88 %. Such high values, however, are not confined to the squalene-containing fish, for there are present in the liver oils of *Notidanus griseus* 13·92, *Acanthias vulgaris* 12·48, *Scymnodon ringens* 33·5 and of *Torpedo nobiliana* 16·63 % of unsaponifiable matter. As to the nature of the substances other than squalene present in these liver oils, reference may be made to the work of Tsujimoto and Toyama [1922] who found 15·42 % of unsaponifiable matter in the liver oil of *Hexanchus corinus*. Squalene was not present, the chief constituents being batyl and selachyl alcohols and these alcohols were present in the liver oils of *Cirrhigaleus barbifer, Somniosus microcephalus, Narcacion takionis, Chimaera owstwonii, C. mitsukurii, Lepidorhinus kinbei and Zameus squamulosus*. Toyama [1924, 1] found these same alcohols present in *Chimaera barbouri* and also reported the presence of chimyl alcohol $C_{19}H_{40}O_3$; they are also

present in the liver oils of a number of other fish [Toyama 1923, 1; 1924, 2]. Further, the presence of oleyl and cetyl alcohols has also been reported, for Toyama [1923, 1] found the unsaponifiable matter of the liver oil of *Chlamydoselachus anguineus* to consist chiefly of oleyl alcohol together with small amounts of cetyl alcohol and cholesterol.

In addition to squalene and the alcohols already discussed, there may be present a saturated hydrocarbon, for Tsujimoto [1917] isolated *iso*-octadecane from the liver oil of *Cetorhinus maximus*, and Toyama [1923, 2] found this hydrocarbon in a number of liver oils.

Another point worthy of notice is that the amount of unsaponifiable matter in any given liver oil may vary considerably. Toyama [1922] reported that the liver oil of *Chlamydoselachus anguineus* contained percentages of unsaponifiable matter varying from 37·06 to 51·65 %; the oil from *Scymnorhinus lichia* yielded 48·51 % while the writer found 81·49 %.

Sufficient has been said to illustrate the interesting problems which are raised by the presence of alcohols and hydrocarbons in such quantities in these fish-liver oils. Evidence concerning the function of these substances and of the chemical processes involved in their utilisation is urgently required, for such evidence may throw fresh light on our conceptions of fat metabolism.

It is of interest at this point to compare the yields of unsaponifiable matter obtained from various mammalian livers expressed as g. % of the liver. Cat 0·32, 0·56, 0·87, 0·38, 0·29; ox 0·35; pig 0·32; horse 0·25; human 0·35, 0·67, 0·58, 0·98; figures for rats' livers have been recorded [Channon, 1926]. These materials were in all cases solids containing much cholesterol, which usually amounted to about 70 % of the total.

### 3. *The proportion of sterol in the unsaponifiable fractions.*

Just as there are wide differences between the Selachii and Teleostei in the amount of unsaponifiable matter in the livers, so are there striking variations in the percentages of sterol present in these fractions. Among the Teleostei, the extreme values for the amount of sterol expressed as a percentage of the unsaponifiable fractions are 42·9 % and 83·5 % with a mean value of 63·3 %. These may appear wide variations but the figures obtained are of the same order, and, since the sterol constitutes a considerable proportion in all these particular unsaponifiable fractions, this mean value of 63·3 % has some significance.

If we consider the oils from the livers of the Selachii, however, a number of curious values obtain for the sterol content, *i.e.* from 0·13 % to 51·45 % with a series of intermediate values. These values appear at first sight to bear no relation to one another, but if we arrange the fish in order so that those with the greatest content of unsaponifiable matter in the liver appear at the top, and also record the percentage of sterol in this unsaponifiable fraction, we obtain the following result (Table III).

Table III.

| Fish | Unsaponifiable matter % | Sterol as % of unsaponifiable matter |
|---|---|---|
| *Scymnorhinus lichia* | 81·49 | 0·13 |
| *Lepidorhinus squamosus* | 72·88 | 0·53 |
| *Spinax niger* | 67·13 | 0·61 |
| *Scymnodon ringens* | 33·5 | 1·47 |
| *Torpedo nobiliana* | 16·63 | 10·59 |
| *Notidanus griseus* | 13·92 | 44·78 |
| *Acanthias vulgaris* | 12·48 | 7·96 |
| *Galeus vulgaris* | 3·82 | 20·46 |
| *Raia clavata* | 2·48 | 24·66 |
| „ *batis* | 2·11 | 51·45 |
| *Scyllium canicula* | 1·91 | 44·70 |
| *Lamna cornubica* | 1·13 | 40·9 |

A curious result is obvious, namely that, in general, as the amount of unsaponifiable fraction of the liver oil increases, so does its sterol content decrease. The only obvious exception in the above table is in the case of *Notidanus griseus*, but as the unsaponifiable fractions were prepared from one sample of oil only in each case, this particular result may be fortuitous. The conclusion from the above table may be expressed in the general form that oils of approximately the same content of unsaponifiable material will contain approximately the same amount of sterol. It is obvious that such a relationship will not be detectable in the unsaponifiable fraction of those livers which contain only 1–2 % of unsaponifiable material. It is thus only among those given, *i.e.* the Selachii, where the unsaponifiable fraction is very high, that the relation becomes apparent, but presumably it is a general one. Such a relationship, in which the amount of sterol present in an oil varies inversely with the amount of the total unsaponifiable fraction of the oil, is one not easy to interpret. It seems less probable to the author that the relationship is one between the sterol and the total unsaponifiable fraction than that it is one between the sterol and the true fat present in the oil.

Further experiments are clearly necessary in which attention must be paid not only to the amount of unsaponifiable matter and sterol in the liver oil, but also the amount of oil yielded by the livers of different fish. The determination of the amount of oil that a given weight of liver contains becomes of importance when it is remembered that the livers of various fish contain markedly different percentages of oil, for although the figures recorded in this paper give the percentages of unsaponifiable matter and sterol in a given liver oil, they do not give any idea as to the total amount of sterol and fat present in the liver itself.

It was stated in the introduction to this paper that one reason for studying the sterol percentages was to obtain light on a possible relationship between squalene and sterol. The evidence given in the above table is quite definite in showing that the very low percentage of sterol in the squalene-containing livers is not due to the presence of squalene as such but to the quantity in

which it is present, for the relationship between sterol and the unsaponifiable fraction is maintained throughout whether squalene is present or not. Thus the low percentage of sterol in the squalene-containing oils is merely due to the mass relations existing between the sterol and the rest of the unsaponifiable fractions, and hence this low percentage of sterol is no evidence whatever of any relation between sterol and squalene.

SUMMARY.

1. A study has been made of the yields of unsaponifiable matter from the livers of a number of fish.

2. The liver oils of the Selachii differ from those of the Teleostei in that, in many cases, very large amounts of unsaponifiable matter occur in the former.

3. A relationship seems to exist between the percentage of unsaponifiable matter in the liver oils of the Selachii and their sterol content. The higher the percentage of unsaponifiable matter in a given oil, the lower is the percentage of sterol in that fraction.

4. Squalene was not detected in the liver oils of any of the fish studied, save in those from three members of the Squalidae family.

5. The question as to whether squalene is synthesised or is derived by the fish from its food is briefly discussed.

The author has pleasure in thanking Dr E. Allen, F.R.S., for the hospitality accorded to him at the Marine Biological Station, Plymouth, and to Mr C. F. A. Pantin and to Mr F. S. Russell for their assistance in obtaining the materials. Prof. I. M. Heilbron kindly supplied some of the oils used.

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# X. THE QUANTITATIVE ESTIMATION OF VITAMIN D.

By HARRY JEPHCOTT and ALFRED LOUIS BACHARACH.

*From the Glaxo Research Laboratory, London.*

(*Received December 9th, 1927.*)

In a recent communication [Jephcott and Bacharach, 1926] we have described a method of detecting vitamin D by its effect on the faecal $p_H$ of rats kept on a rachitogenic diet. In the curves shown in that paper there were indications of a quantitative relationship between the amount of vitamin and the extent of the change in $p_H$.

A series of doses of one batch of "ostelin" (an antirachitic extract of cod-liver oil prepared by a method based on that described by Zucker [1922]) has been fed to pairs of animals under conditions described in our previous communication. The results are shown in Fig. 1, curves *A* to *E*.

According to our custom, mixed samples of faeces were taken from groups of four animals until the beginning of the administration of the substance under test; the animals were then divided into two pairs, and the samples collected from these pairs. In the curves of Fig. 1 the preliminary period samples were collected together from groups A and B and from groups C and D; all the eight animals in those four groups were, it so happens, males from one litter. In the case of group E, the samples for the preliminary period represented those of the two animals used subsequently and two other animals used for a different test, all the animals in this case being females. The olive oil used for solution was entirely without effect on the faecal reaction of the animals, as has been found from the fact that tests of inactive substances dissolved in this oil have given quite negative results; the difference between curve *D* (four drops of olive oil) and curve *E* (one drop of olive oil) also bears this out.

The results of the experiments can be expressed in a slightly different manner. If the lowest value for the mean of two consecutive daily readings of faecal $p_H$ ("the minimum mean $p_H$") be plotted against the actual weight of vitamin-containing substance administered, a result is obtained which is shown in Fig. 2. Without wishing to draw too rigorous conclusions from a single series of observations on animals, however consistent, we think it justifiable to point out that the position of the values plotted suggests that they fall on an "S"-shaped curve, which is what might be expected from general considerations of the method of action of vitamin D. It would appear

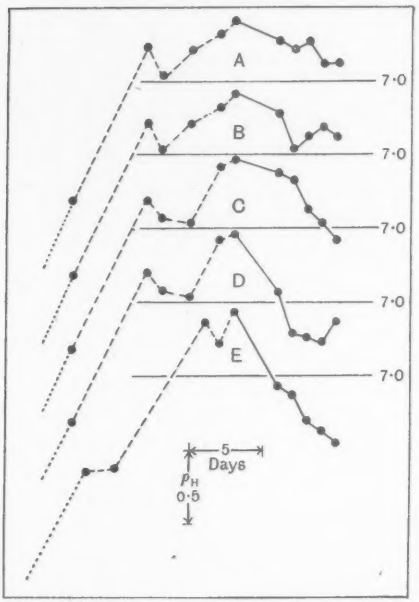


Fig. 1. Curve A. 0·013 mg. "ostelin" dissolved in 22·5 mg. olive oil.
Curve B. 0·026 mg. "ostelin" dissolved in 45 mg. olive oil.
Curve C. 0·039 mg. "ostelin" dissolved in 67·5 mg. olive oil.
Curve D. 0·053 mg. "ostelin" dissolved in 90 mg. olive oil.
Curve E. 0·066 mg. "ostelin" dissolved in 22·5 mg. olive oil.
..... Basal diet only: no readings taken.
– ● – – ● – Basal diet only: readings taken.
—●—●— Basal and supplementary diets: readings taken.

that in this test the minimum amount of vitamin D which shows any appreciable effect is that which gives a lowering of faecal $p_H$ to 7·1 and that the maximum effect is produced with an amount of vitamin D which gives a lowering of the faecal $p_H$ to about 6·6. In our previous communication we wrote: "...until two consecutive readings give a mean value of $p_H$ 6·7 or less, and neither more than 6·8. For our own laboratory convenience we have considered the quantity of antirachitic substance necessary for this purpose to be an antirachitic unit." Since then, we have found it convenient to subdivide this unit into ten. There are about 100 such units in a gram of the most active cod-liver oils.

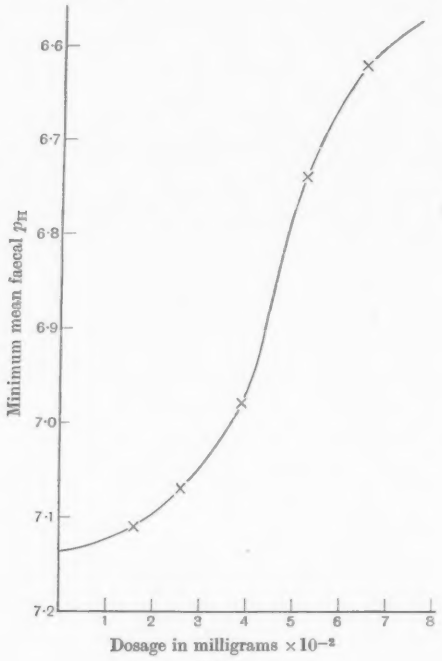


Fig. 2.

SUMMARY.

Evidence is submitted that the extent of the lowering of faecal $p_H$ by the administration of vitamin D to rats on a rachitogenic diet is a function of the amount of vitamin D administered.

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# XI. NOTE ON THE RELATION BETWEEN (*a*) EXTERNAL HYDROGEN ION CONCENTRATION AND (*b*) THALLIUM SALTS, AND THE RATE OF AMPHIBIAN METAMORPHOSIS.

By J. BELEHRADEK, JULIAN SORELL HUXLEY and FRANCIS ROY CURTIS.

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(*Received December 10th, 1927.*)

(*a*) External $p_H$ and metamorphosis (J. B. and J. S. H.).

Recent work by Scheer [1926] has demonstrated that frog tadpoles grow more rapidly in alkaline than in acid media between $p_H$ 5·5 and 8·2. On the other hand, Jewell [1920] has found that respiration in tadpoles is markedly increased when the liquid of the culture is made slightly alkaline. It seems then that slightly alkaline media increase the metabolic rate of tadpoles, which is in agreement with facts found in lower animals.

Amphibian metamorphosis is accompanied by an increase of metabolism [Belehradek and Huxley, 1927]. We have accordingly thought it of interest to investigate whether variations of the external $p_H$ may exert an influence on the course of amphibian metamorphosis. The method adopted was as follows.

Series of glass jars of uniform size were arranged in a tank through which tap-water circulated in order to keep the temperature low and uniform. Each jar contained 200 cc. of tap-water to which 40 cc. of $M/5$ phosphate mixtures of various $p_H$ were added. We used these rather high concentrations to maintain the buffering power of the media. The $p_H$ of the contents of the jars was measured from time to time by means of indicators. In various experiments three, four or six different media were employed, selected from the series of $p_H$ 6·8, 7·0, 7·2, 7·5, 7·8, and 8·1. Lots of five or ten *Rana temporaria* tadpoles were put into each jar and fed daily with small amounts of veal. Care was taken to ensure that each lot was similar in regard to the sizes of the tadpoles. Feeding with green algae was found unsatisfactory because of variations in $p_H$ produced by their activity. The solutions were changed every day, or every two days.

Metamorphosis was induced by means of thyroid in three series, and by free iodine in another three series. In the thyroid experiment, thyroid solution, prepared from Armour and Co.'s tablets, was administered for a period not

exceeding 24–36 hours. In the iodine series, one or two drops of a weak alcoholic solution of iodine (saturated alcoholic solution diluted in proportion 1 : 40) were added every day to each vessel. Thyroid administration of course produces a more explosive metamorphosis, while that due to iodine is much more gradual.

The following table shows the results obtained in one of the thyroid series.

| No. | $p_H$ of phosphate stock solution used | $p_H$ of medium during experiment | Metamorphic changes 18. viii | Metamorphic changes 22. viii |
|---|---|---|---|---|
| 32 | 6·6 | 6·8 | none | none |
| 33 | 7·5 | 7·5 | none | 3 out of 9 with fiddle-shaped bodies |
| 34 | 7·8 | 7·7 | none | 5 out of 8 with fiddle-shaped bodies |
| 35 | 8·1 | 8·0 | beginning of tail reduction | All individuals metamorphosing, marked reduction of tail, 1 tadpole with fore-limbs |

On 13. viii, a thyroid extract was made with the equivalent of 0·5 g. of dry gland to 20 cc. of water. 2 cc. of this extract were added to each vessel, producing a solution equivalent to one part fresh gland in 960 of solution, and the animals were kept in the mixture for 24 hours, before being placed in the buffered solutions.

Similar effects were observed in all thyroid series, although in that given above the results were the most definite. Probably the comparatively weak dose of thyroid chosen in this series was the most favourable. With free iodine we have so far not been able to detect any regular and definite differences between animals kept at different $p_H$.

Vital staining with a 1 : 100,000 solution of neutral red showed that the $p_H$ of the tadpoles' tissues (margin of the tail-fin) is about the same in acid as in alkaline media. This is not surprising, as it is known that living cells do not change their internal $p_H$ when the acidity or alkalinity of the medium is altered [see Pantin, 1923] and, on the other hand, marked differences in physiological functions of the organism may be induced by varying the external $p_H$, without any accompanying change of the internal $p_H$ of the organism.

Our experiments therefore demonstrate that the rate of metamorphosis, induced by keeping frog tadpoles in weak thyroid extracts, depends on the $p_H$ of the medium, being, between $p_H$ 6·8 and 8·0, greatest on the alkaline and least on the acid side. This effect is not connected with variations of the internal $p_H$. In tadpoles treated with free iodine no definite effects of different external $p_H$ could be detected.

(*b*) THALLIUM ACETATE AND METAMORPHOSIS (J. S. H. AND F. R. C.).

Of recent years the use of thallium acetate for epilation has been revived, as it is a much more convenient and less dangerous method of epilating young children than the employment of X-rays.

Arising from the experimental study of the element in its action on animals the suggestion was made by Buschke and his collaborators [1926] that the epilating effect is due partly to an effect on the sympathetic nervous system and partly to an effect on certain endocrine organs, especially the thyroid, adrenal, and testis. Furthermore, they stated that the substance had an inhibitory effect on the metamorphosis of amphibian larvae.

If thallium produces this effect by a direct depressant action on the thyroid gland, as is suggested by the above-mentioned workers, then we thought it might be possible to demonstrate a mutual antagonism between thallium on the one hand, and thyroid extract and iodine on the other hand, when young tadpoles were exposed to different concentrations of these substances.

Tadpoles, about half-grown, of approximately the same degree of development, were selected, divided into equal groups, and exposed to varying dilutions of thallium acetate in tap-water. After preliminary experiments, the dilutions $1/10^5$, $1/2 \times 10^5$, $1/4 \times 10^5$ and $1/8 \times 10^5$ were chosen, with $1/16 \times 10^5$ and $1/32 \times 10^5$ in addition in the first experiment. Half the tadpoles used were placed in 1/1000 thyroid extract for 24 hours before they were transferred to the thallium solutions; the other half, in addition to their exposure to thallium solution received two drops every other day of a saturated solution of iodine in alcohol diluted 40 times with distilled water. Controls were also kept consisting of tadpoles in tap-water only, tadpoles in iodine and tap-water only, and thyroid-treated tadpoles in tap-water.

$1/10^5$ thallium acetate solution is fatal to tadpoles in from 24 hours to 6 days. It is interesting to note that the fatal effect appears to be retarded though not prevented by previous thyroid treatment. For instance, the iodine-treated specimens were dead in 3 days, but the thyroid-treated specimens not till 6 days. In previous experiments this concentration had been found to kill tadpoles without iodine or previous thyroid treatment in 1 to 3 days (at approximately the same temperature).

In dilutions of $1/2$ and $1/4 \times 1/10^5$ the mortality was slightly higher than in lower concentrations or in controls, but this may not have been significant.

The results of all experiments were concordant in showing no antagonism between thallium and the metamorphic action either of iodine or thyroid. As a matter of fact, in the first thyroid series the tadpoles in $1/2$ and $1/4 \times 1/10^5$ thallium acetate showed a slight acceleration of metamorphosis as compared with controls and those in higher dilutions. In the second series the numbers were not sufficient to permit a decision to be made at this point. Considerable numbers of tadpoles would be needed to establish the reality of this slight adjuvant action of thallium on metamorphosis, and unfortunately at the time (July) no further specimens were to be obtained.

The result seems clearly to indicate that there is no antagonism between thallium and the metamorphosis-accelerating function of foreign thyroid or of the tadpoles' own thyroid (iodine series) under the conditions of these experiments. It may be added that unpublished experiments by one of us (F. R. C.) indicate a similar conclusion regarding the prevention of thallium epilation in rats by feeding thyroid extract. The animals were found to epilate as early and as completely as rats not fed on thyroid extract.

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# XII. THE VITAMINS OF ORANGE JUICE.

By STANLEY GORDON WILLIMOTT.

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(*Received December 12th, 1927.*)

A study of the literature leads to the conclusion that the value of orange juice as an antiscorbutic has tended to obscure the fact of the presence of some other important factors.

For example, less than a decade ago the presence of vitamins other than vitamin C was apparently unsuspected; and Drummond and Coward [1920], who used this product in their basal diet for titrating vitamin A, believed it to be devoid of vitamin A on the evidence of direct tests. Although at the level fed (5 %) it may not have exerted a serious vitiating effect in their experiments, it is perhaps significant that later [Drummond, Coward and Handy, 1925], orange juice was replaced by lemon juice in their basal diet.

Osborne and Mendel [1919, 1920] were the first to show that orange juice contained appreciable amounts of vitamin B, and that 10 cc. was an adequate dose for the rat. This finding was substantially confirmed by the clinical work of Byfield, Daniels and Loughlin [1920] on marasmic infants. Subsequently, Osborne and Mendel [1921] demonstrated the presence of vitamin A in the juice and showed that 10 cc. was an adequate dose for the rat. The presence of these two vitamins in orange juice has since been confirmed by several different investigators [Hess, McCann and Pappenheimer, 1921; Coward, 1923].

The present investigation was undertaken with the object of determining as accurately as possible, under carefully controlled conditions, the minimum adequate dose of fresh orange juice of known origin as a source of vitamin A and vitamin B respectively for the rat. Because of the fact that the fat-soluble vitamins had not then been differentiated, the supply of vitamin D in testing for vitamin A has not been controlled in previous investigations of the orange. Moreover, in these studies the species of orange employed has seldom been stated, variations in the constituents of the juice of different varieties apparently being neglected [cf. König, 1903].

The antisterility vitamin E content of orange juice has been investigated by Evans and Burr [1927]. Doses of 16 cc. of orange juice (origin not stated though presumably California) per head per day were fed to sterile rats. From their findings, Evans and Burr concluded that orange juice is a comparatively low source of vitamin E. Their results showed that, while generally impotent to effect cures of this dietary sterility, orange juice succeeded in a low proportion of cases.

The antirachitic value of orange juice has been little studied and it seemed worth while to investigate this point also.

On the other hand, orange juice has been exhaustively investigated for its antiscorbutic potency and it has been shown by Goss [1925], using the fresh juice of the same species of orange (California), that the minimum daily dose for the guinea-pig is 1·5 cc. Work on vitamin C was therefore omitted. The effect of concentration on the vitamins of orange juice was also not investigated on this occasion but it is hoped to publish a further paper on this question.

A preliminary note of some of the results which follow was communicated in a paper read before the British Pharmaceutical Conference [Willimott, 1927].

## Experimental.

The orange juice employed throughout this work was obtained from ripe California oranges (Sunkist brand). It was hoped to complete the whole of the experimental work using the juice of navel oranges solely, but, as the feeding experiments became protracted beyond the natural season (December to May), it was necessary to use also some Valencia oranges from the same source.

On arrival in the laboratory each consignment of fruit was carefully sampled, and the juice actually used in experiment was representative of about 600 "sunkist" oranges. Individual oranges were taken as required from samples which were laid out on paper in a room, the temperature of which ranged between 3° and 7°. In preparing the material for experiment the selected oranges were first peeled in order to obviate any possible contamination of the juice by the rind oil, which is now known to be rich in vitamin A [Morgan, 1923]. The orange pulp was then well pressed out on a Büchner funnel and the juice filtered through muslin. For chemical analysis the juice was refiltered through filter paper yielding a product free from cell tissue. The results of the analyses of California navel and Valencia orange juice, together with figures given by König [1903], for California navel orange juice, are summarised in Table I.

### *Methods.*

Inbred Wistar albino rats were used throughout the work, the curative method of testing being adopted. In titrating vitamin A the vitamin D requirement was supplied by a suspension of 2 % irradiated cholesterol in liquid paraffin. Stoppage of growth and onset of xerophthalmia were used as the criteria for absence of vitamin A. The appropriate dose of orange juice was then administered without delay. The food intake was observed throughout. In the plan of the work exploratory groups were first arranged in order to determine approximately the minimum adequate dose of orange juice for each of the respective vitamins. Confirmatory groups were then set up to verify these preliminary findings.

The method of dosing found to be most satisfactory was to give the juice separately in advance of the basal ration. The measured doses were placed in

Table I. *Analyses of California oranges.*

| | Navel | Valencia | Navel (König) |
|---|---|---|---|
| Tintometer reading | 39·0 yellow units | — | — |
| ($\frac{1}{2}$″ cell) | 5·8 red ,, | — | — |
| | 3·8 blue ,, | — | — |
| Peroxidase tests | negative | negative | — |
| Reaction ($p_H$) | 4·2–4·6 | 4·2 | — |
| Specific gravity at 15° | 1·0509 | 1·0498 | — |
| | % | % | % |
| Reducing sugar (before hydrolysis) | 5·3 | 5·3 | *4·08 |
| Reducing sugar (after hydrolysis) | 10·85 | 9·8 | 10·03 |
| Total solids | 12·08 | 11·6 | 13·00 |
| Total N | 0·103 | 0·149 | — |
| Protein (N × 6·25) | 0·644 | 0·929 | 1·27 |
| Ash | 0·44 | 0·47 | 0·42 |
| Fat | 0·10 | — | — |
| Acid (citric acid) | — | — | †0·97 |
| Average wt. of oranges | 200·3 g. | 198·4 g. | 307·4 g. |
| Peel (flavedo and albedo) | 29·3 % | 26·7 % | 28·5 % |

The data given in column 3 are taken from König [1903] and are the average of six analyses. The protein figure was given under the heading "nitrogenous substance," and the factor employed in computing it was not stated.

The enzyme tests were carried out by the guaiacum, benzidine and α-naphthol tests.

The hydrolysis of the sugars was carried out with 1 % HCl at 40°. Qualitative analysis of the ash of navel and Valencia orange juice indicated the presence of calcium, magnesium, phosphorus, iron and silica.

Quantitative analysis of the ash of California navel orange juice is given by König. Iron, aluminium, manganese, calcium, magnesium, potassium, sodium, phosphorus, sulphur, silica and chlorine were estimated. Of these potassium was richest.

* Refers to cane-sugar.

† For California navel orange juice, this figure is low and was probably obtained at the end of the season on very ripe fruit.

flat-bottomed pots which could not be turned over by the animals, each of which occupied a separate cage during the period of dosage. It was soon found that rats vary considerably in the ease with which they consume orange juice. However, doses up to 7·5 cc. were consumed completely without difficulty, and as soon as the animals had become accustomed to it, doses of 10 cc. also. Preliminary experiments showed that doses higher than 10 cc. are not easily taken by young rats on a synthetic diet. On the other hand, adult rats on a stock diet were found to consume up to 20 cc. per head per day, the animals exhibiting a keen liking for the juice. The problem of accurate dosage, especially in the case of vitamin A, was thus rendered comparatively easy.

### *Vitamin A.*

The exploratory group consisted of six young rats which were placed on the diet deficient in vitamin A usual to this laboratory [see Willimott, 1926]. Vitamin A deficiency, as evidenced by weight decline and xerophthalmia, became established within 4–6 weeks (see Fig. 1).

Two rats now received 10 cc. of the orange juice per head per day, three others 7·5, 5·0, 2·5 cc. respectively and one served as a control which received no orange juice. The weight changes resulting from the consumption of these different doses throughout a period of about five weeks are shown in Fig. 1.

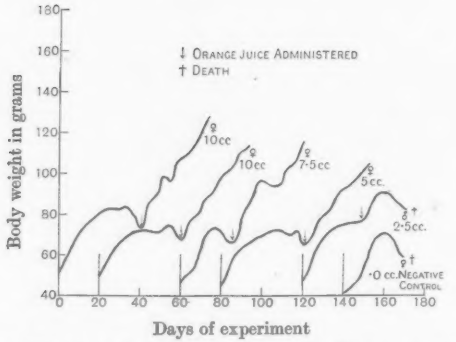


Fig. 1. Vitamin A in California orange juice. Exploratory groups.

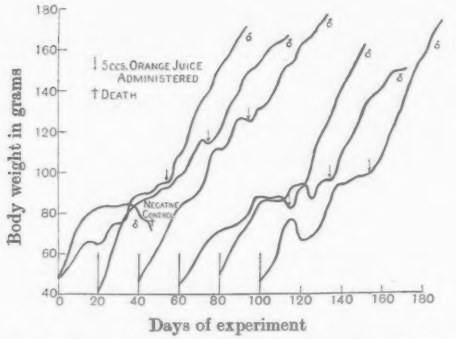


Fig. 2. Vitamin A in California orange juice (male rats).

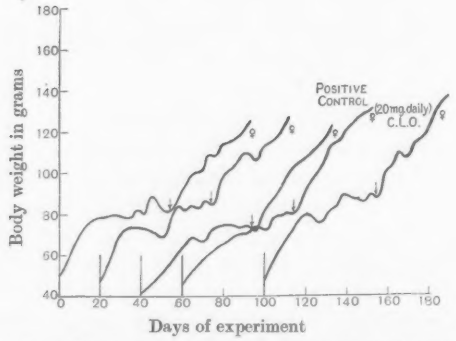


Fig. 2*a*. Vitamin A in California orange juice (female rats).

Rats receiving doses of 5 cc. or more all responded promptly; there was little difference between them in growth rate, and they were all in good physical condition when observations were discontinued. The animal receiving 2·5 cc. per day died in the third week after the commencement of dosage. Xerophthalmia was not present, but autopsy revealed symptoms of vitamin A deficiency. The negative control died on the 29th day of the experiment, xerophthalmia being present. During the period of dosage the food intake rose from an average of 5 g. per head per day to 10 g. for each surviving animal at the end of the experiment. From these experimental data it was inferred that 5 cc. was probably the minimum adequate dose of orange juice as a source of vitamin A.

It now remained to confirm or modify this finding, and to this end a group of 12 young rats (for sexes see Figs. 2 and 2 *a*) was restricted to the same vitamin A-free diet. By the 8th week the weights of all these animals had become stationary or declining, while incipient xerophthalmia was evident. At this point the food intake averaged about 8 g. per head per day. Positive and negative controls were employed, the positive control receiving about 20 mg. of a potent Iceland cod-liver oil per day; the negative control died on the 47th day of experiment with symptoms of severe vitamin A deficiency as indicated by xerophthalmia, congestion of the lungs, hypertrophy of the suprarenals, and thinning of the gut.

To the remainder of the group 5 cc. of California navel orange juice was now fed daily to each animal for a period of 5 weeks. Prompt resumption of growth, especially marked in the females (see Fig. 2 *a*), and disappearance of xerophthalmia were observed. The average gain in weight per week was 12·5 g. in the males and 8·5 g. in the females as compared with 10 g. in the female control receiving cod-liver oil. The average food intake also rose from 8 g. to 11·5 g. per day. As the group had thus responded satisfactorily the dose of 5 cc. was not disturbed. From the time dosage commenced, lemon juice was omitted from the basal diet, the orange juice supplying vitamin C as well as the necessary vitamin A. At the end of the experiment there was no noticeable difference in physical condition between the positive control and the remainder of the group receiving orange juice. Autopsies on male and female rats including the control revealed normal physiological conditions, the presence of fat in the viscera being noteworthy. It appeared therefore, that 5 cc. of the California navel orange juice employed contained an adequate supply of vitamin A for the rat.

Confirmation of the presence of vitamin A was next sought by application of colour tests. 125 cc. of the orange juice were concentrated *in vacuo* to a golden coloured syrup which was well extracted with chloroform and the pigment removed by charcoal (Merck's) in the cold, following the technique recently described by Willimott and Wokes [1927]. The reactions with arsenic and antimony trichlorides were definitely, if not strongly, positive; the results with concentrated sulphuric acid and phosphorus pentoxide were doubtful.

*Vitamin B.*

In the work on this vitamin, curative experiments were employed, while growth promotion in rats was used as the criterion for vitamin B; but it was not possible to test the antineuritic value of the juice on pigeons on the lines indicated by the recent work of Chick and Roscoe [1927]. In view of the fact that the minimum curative dose of orange juice was to be determined as accurately as possible, special pains were taken, *e.g.* by the omission of lemon juice, to obtain a basal diet free from vitamin B. Even so, in preliminary

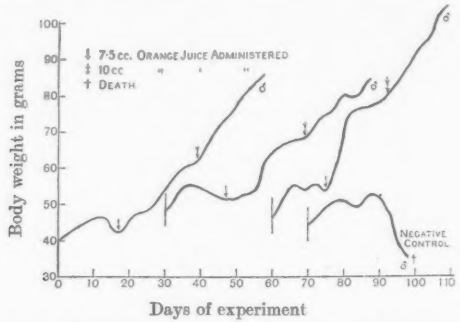


Fig. 3. Vitamin B in California orange juice (male rats).

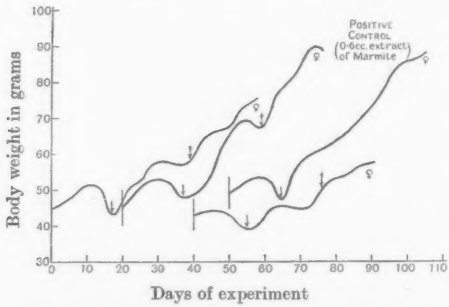


Fig. 3*a*. Vitamin B in California orange juice (female rats).

experiments the potato starch, which has long been employed in the vitamin B-deficient diet of this laboratory, was found to be contaminated with this vitamin. It was therefore replaced by rice starch and then, in fresh experiments, failure in the normal period (cf. Figs. 3 and 3 *a*) was secured. One result of this improved technique was seen in the necessity for raising the daily addendum of an extract of marmite from 0·25 cc. to at least 0·6 cc. in order to obtain normal growth.

This is so much higher than the usual figure that it has led to further experiments on the vitamin B content of yeast extracts prepared under different conditions, details of which it is hoped to publish later.

Preliminary experiments, using California Valencia orange juice, showed that doses of 5 cc. were clearly insufficient to supply rats with their requirement of this vitamin. To economise space these curves are not given. A large group of eight young rats, consisting of equal numbers of male and female, was now restricted to the vitamin B-free diet. Within a fortnight the group had declined in weight and some of the animals showed signs of paralysis in the hind legs. A daily dose of 7·5 cc. of the fresh juice was now administered to each of six of the group, the remaining rats acting as positive and negative controls (see Fig. 3). The positive control received the equivalent of 0·6 g. of marmite extract. The negative control died on the 27th day of experiment with the usual signs of severe vitamin B deficiency. Most of the group responded quickly to the dosage of orange juice which was completely ingested by all except two animals whose average consumption was 6·5 cc. per day. At the time dosage commenced, the average food intake was only 4·5 g. per day, the animals showing little inclination for food. After receiving orange juice, however, the appetite improved and the food intake rose steadily up to 8 g. at which it remained for the rest of the experiment. In the third week after administering orange juice there was a tendency for growth to fall off, although the group as a whole was in good health. On the 23rd day after orange juice was first administered the dose was therefore raised to 10 cc. daily. With the majority of the group it appears that there was a definite growth response to this addition (Fig. 3).

Satisfactory growth was maintained for a further 17 days up to the conclusion of the experiment. During this period there was little difference in the weekly growth increase between the control and the remainder of the group receiving orange juice (7·2 g.). There was no sign of nervousness in these animals, their coats were sleek, and they were of gentle demeanour. The male and female rats autopsied were normal. From these findings a dose of 10 cc. of Valencia orange juice was considered to be adequate for the vitamin B requirements of the rat.

*Vitamin D.*

From knowledge of the formation of the calcification-promoting vitamin D by light rays it might be anticipated that this factor would be present in a fruit such as the orange. However, the experiments so far carried out have not realised this supposition.

The method employed was a modified Zucker technique, in which the faecal reaction is made alkaline by feeding a rachitogenic diet low in fat; if an antirachitic substance is now fed, the faecal reaction becomes acid within 3 or 4 days [Jephcott and Bacharach, 1926]. Persistence of alkalinity of faecal $p_H$ is regarded as indicative of the absence of vitamin D. However, in applying the method to orange juice a number of precautions are called for. Orange juice, which is naturally fairly acid ($p_H$ 4·5), might of itself lower the $p_H$, irrespective of the presence of any vitamin D. This point was investigated by

Redman, Willimott and Wokes [1927] who found that ripe California navel orange juice was so well buffered that 12 to 15 cc. of normal NaOH were required to bring 100 cc. to $p_H$ 7·0. Such neutralised orange juice was shown to have no effect upon the alkalinity of reaction of the faeces of rats on Zucker's "low fat" diet. It was now decided to test the fresh un-neutralised orange juice direct. Four young albino rats were placed on Zucker's diet, vitamin A being supplied by 2 g. of fresh spinach leaves. Within 10 days the faecal reaction, which was estimated both electrometrically and colorimetrically, had become alkaline. Doses of 10 cc. per day of fresh navel orange juice were now fed separately as in the previous experiments, and the dose was usually consumed completely. Since this amount of orange juice was known to contain an ample supply of vitamin A for rats, the addition of spinach leaves was discontinued. After 2 weeks' administration of the juice the faecal reaction of each animal was still definitely alkaline. The group was now divided, one half receiving a known supply of vitamin D as Iceland cod-liver oil in addition to the orange juice, the remaining half continuing as before acted as a control. The faecal reaction of the cod-liver oil rats now became acid within 4 days, and remained so with slight fluctuations so long as the oil was fed.

The alkalinity of the faeces of the control rats on orange juice alone during the same period remained undisturbed. From some experience of the method, these results seem to me to afford presumptive evidence at least of the absence of vitamin D from orange juice.

However, it is obviously desirable that the problem should be much more completely investigated, and it is hoped later to do this.

## Discussion.

While the fact of the presence of vitamins A and B in fresh orange juice of known origin has been confirmed under carefully controlled conditions, it has been further demonstrated that, at any rate in the case of California orange juice, it is more potent in vitamin A than has previously been supposed. Osborne and Mendel's adequate dose for the rat was 10 cc. for this vitamin [1921], and later the same dose of orange juice was used with success by Coward [1923]. However, at the time Osborne and Mendel first adduced experimental evidence for the presence of vitamins A and B in orange juice (origin not stated), the fat-soluble vitamins had not been differentiated. It is possible that the supply of vitamin D in the present investigation was in part responsible for the finding that half Osborne and Mendel's dose was adequate in its supply of vitamin A for good growth and cure of xerophthalmia in the rat. At the same time possible differences in composition of the orange juice used must be borne in mind.

In work on vitamin B, the loss of appetite characteristic of this deficiency is a difficulty rendering the interpretation of the results less easy. However, it is probably safer to accept 10 cc. rather than 7·5 cc. as the minimum adequate dose of orange juice for the supply of vitamin B, in agreement with Osborne

and Mendel [1920]. Byfield, Daniels and Loughlin [1920], however, apparently found 7·5 cc. to be adequate in vitamin B for growth, but Stammers [1924], who fed 10 cc. doses of orange juice to rats (which were not young), concluded that its content of vitamin B was insufficient for normal growth. His findings are in conflict with those of other investigators. The vitamin content of the juice and other tissues of the orange and other *Citrus* fruits is summarised in a table by Willimott and Wokes [1926].

Although the work on vitamin D is incomplete, the results obtained by the Zucker method appear to indicate the absence of this factor from orange juice. This result if confirmed is of obvious importance in clinical medicine, especially in view of the work of Chaney and Blunt [1925]. On the addition of 20–23 oz. daily of California Valencia orange juice to the diet of growing children, these authors observed an increased retention of calcium, a more marked retention of phosphorus, a smaller retention of magnesium, and a positive nitrogen balance. It seems possible that the alkaline nature of the ash of orange juice, which remains after combustion in the body, has some important bearing on the cause of the marked retention of the bone-forming minerals. The favourable influence of orange juice upon growth is a problem for further investigation.

As early as 1919 the absence of vitamin D from orange juice was indicated by Mellanby [1919, 1920] who found that a quarter of an orange per day did not prevent rickets in puppies. Later, Korenchevsky and Carr [1924] compared the value of orange juice (origin not stated) with decitrated lemon juice in the calcification of the skeleton of rats, and concluded that neither had any definite influence. Clinical experience is also in harmony with the laboratory results, in that it is known that children can develop rickets even when taking appreciable amounts of orange juice. Lastly, Maslow, Shelling and Kramer [1926] have obtained some interesting results with a commercial preparation, "Mission orange juice," both in the raw state and after irradiation by the quartz mercury vapour lamp. The dose fed was 15 cc. per head per day for periods ranging from 7 to 15 days. Using McCollum's "line test," these authors showed (1) that the non-irradiated product had no calcifying effect on the metaphyses of rachitic rats, (2) that the irradiated product produced healing which was almost complete at the end of 15 days.

In interpreting these results it must be remembered that "Mission orange juice" of commerce is a sweetened bottled product, and these results would have had more significance had the work been carried out on fresh orange juice. Moreover, in producing vitamin D by irradiation, the question of the possible parallel destruction of vitamin A deserves careful consideration.

SUMMARY.

1. Orange juice from a known source (California navel and Valencia, Sunkist brand) and representative of 600 fruits was used throughout the work. Complete analyses were made (Table I).

2. Working under carefully controlled conditions, 5 cc. of navel orange juice were found to contain a sufficiency of vitamin A for growth and well-being in the rat.

3. 10 cc. of Valencia orange juice appeared to be adequate for the vitamin B requirements of the rat.

4. No evidence was found for the presence of vitamin D in navel orange juice.

I am indebted to Sir F. Gowland Hopkins for his advice and support, to Mr F. Wokes for his interest in the work, and to the California Fruit Growers Exchange, who kindly supplied the Sunkist oranges used in this investigation.

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# XIII. NOTE ON THE UNSAPONIFIABLE MATTER FROM THE STOMACH OIL OF *SCYMNORHINUS LICHIA.*

By EDWARD DAVID KAMM.

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(*Received December 13th, 1927.*)

In a previous communication [Heilbron, Kamm and Owens, 1926] it was reported that oil had been obtained from the stomach of the elasmobranch fish *Scymnorhinus lichia*, and that this oil contained the hydrocarbon squalene.

An investigation of the oil has now been made in some detail.

This fish, known to the fishermen as "Darkie Alf," is commonly spoken of by them as having an oil bag in the stomach, and the oil is at times used by them as fuel for their lamps.

The specimens here to be described were in excellent condition, and averaged 1 m. in length and 5 to 6 kg. in weight. Before dissection, the pylorus was ligatured, and the stomach was then removed. This was in all cases distended, sometimes very largely so, and was full either of clear oil, or, if the fish had been feeding just before capture, of an oily mass of undigested food. The content of oil was usually about 250 cc. As will be seen below, this oil is almost identical with the liver oil, and no entirely satisfactory explanation of its function is yet forthcoming.

While it has only been possible to obtain specimens during three months of the year (March to May), it is to be noted that stomach oil was present in every member of the species examined, while members of other closely related species of shark or dog-fish contained no readily recognisable quantities of oil in their stomachs.

It is tentatively suggested that some of the liver oil may have come into, and been temporarily stored in, the gall bladder, whence it may have been forced through the bile duct into the small intestine and hence into the stomach. Whether this process is a normal one of the living animal or takes place *post mortem* on the release of pressure consequent on the sudden ascent from its normal habitat of more than 100 fathoms to sea level, must still be a matter for conjecture.

## Examination of the Oil.

*Extraction and examination of the unsaponifiable matter.*

The oil (2065 g.) from the stomachs of 9 fish was subjected to distillation at a pressure of 2 mm., whereby 1000 g. of a liquid boiling at 228–230° were obtained. This had $n_D^{20°}$ 1·4967, $d_{20°}^{20°}$ 0·8536, and on treatment in ether solution with gaseous hydrogen chloride, gave a quantitative yield of squalene hexahydrochloride (M.P. 112–120°) [cf. Heilbron, Kamm and Owens, 1926].

The residues were then saponified by boiling with 5000 cc. of 10 % alcoholic NaOH for 8 hours. The alcohol having been distilled off, the soap solution was mixed with an equal weight of fine silver sand, and was taken to dryness on the steam-bath. The dried soaps were exhaustively extracted with acetone, the acetone extract concentrated by distillation, and the concentrate dissolved in ether, washed with water until neutral, and dried.

After removal of the solvent, the residue was now again distilled *in vacuo*, a fraction B.P. 210–233°/3–4 mm. being removed. This was shown, by the hydrochloride test, to be almost pure squalene.

The residue (50 g.) was resaponified with an alcoholic solution of sodium ethoxide, the neutral portion (15 g.) being again extracted with ether. Distillation of this yielded an oil (B.P. 190–225°/0·55 mm.) which was not further separable by fractional distillation. A small sample taken at this point showed a negative digitonide test for cholesterol.

After slight dilution with pentane, the whole fraction was allowed to stand undisturbed in the ice-chest for several weeks, when a white crystalline solid started to separate, and was filtered off from time to time. This was recrystallised four times from absolute alcohol, 3 g. of pure white crystals being obtained. These were alcoholic in nature, and were saturated to bromine solution. They melted at 67°, and when mixed with pure batyl alcohol at 67·5°; while pure batyl alcohol melted at 69°. C = 72·4, 72·3, H = 12·6, 12·6 % ($C_{20}H_{42}O_3$ requires C = 72·7, H = 12·7 %) [cf. Tsujimoto and Toyama, 1922; Toyama, 1924; Weidemann, 1926].

A small portion of the residue, which could not be induced to crystallise further, was now again tested for squalene, and was found to contain only 9 % of this compound.

The remainder (9 g.) was therefore dissolved in 100 cc. of ethyl acetate and was hydrogenated, a rapid stream of hydrogen being passed through it at 18° for 7 hours in the presence of a palladium catalyst supported on Kahlbaum's charcoal. After removal of the catalyst by filtration and of most of the solvent by distillation *in vacuo*, a further quantity of batyl alcohol separated, showing that in all probability selachyl alcohol ($C_{20}H_{40}O_3$) had also been present. This result was to be expected since Tsujimoto, Toyama and Weidemann have shown that this alcohol usually occurs associated with batyl alcohol.

The constitution of the unsaponifiable matter may therefore be summarised as follows:

| | |
|---|---|
| *Unsaponifiable matter* | |
| Squalene (by distillation) | 1000 g. |
| Residue (by saponification) | 45 g. |
| | 1045 g. = 50·6 % of original oil |
| This consists of | |
| Squalene (distillation) | 1000 g. |
| (distillation of unsap.) | 23 g. |
| (estimated as present in residue) | 1 g. |
| | 1024 g. = 98 % |
| Batyl alcohol | 3 g. = 0·28 % |
| Residue (chiefly selachyl alcohol) | 8 g. = 0·96 % |

In conclusion I desire to thank Profs. I. M. Heilbron and J. Johnstone for advice and help, Dr W. M. Owens for a pure specimen of batyl alcohol, and the Food Investigation Board of the Department of Scientific and Industrial Research for a grant which enabled the research to be undertaken.

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# XIV. THE RANCIDITY OF COCONUT OIL PRODUCED BY MOULD ACTION[1].

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(*Received December 14th, 1927.*)

It is now generally agreed that the deterioration of oils and fats, whether due to the action of air, light and moisture, or to the action of micro-organisms, is an oxidative process subsequent to partial hydrolysis. In these two forms of rancidity—that of pure fats (where the absence of nidus prevents bacterial or mould growth) and that caused by micro-organisms—the course of the oxidation is not the same. In the latter form the combustion is probably more vigorous and supplies energy to the organism. Much has been written on the deterioration of pure fats, a summary and bibliography being given by Hepburn [1909]; but on biologically produced rancidity comparatively little work has been done, that of Jensen [1902] being probably the most important.

As it contains only a small proportion of unsaturated glycerides, coconut oil does not quickly deteriorate when exposed to atmospheric conditions, but eventually acquires the characteristic odour of all fats under these conditions. When subjected to mould action, however, it gives rise to a pungent "perfume" or ester-like odour quite different from that already noted. This has been observed, but not identified, by several investigators [Biffen, 1899; Walker, 1906; Jacobsen, 1918]. Biffen thought, erroneously, that it was amyl butyrate, but the author [1921] has shown it to be due to the formation of methyl ketones; Stärkle [1924] arrives at similar results and suggests that ketones are formed in a way analogous to Dakin's synthesis of methyl ketones from fatty acids [Dakin, 1910]. Derx [1925] confirms the author's results and his contention that *Oidium lactis* does not produce ketonic rancidity, and finds further that some *Penicillium* species produce ketonic or non-ketonic rancidity according to the composition of the medium.

The formation of methyl ketones in the decomposition of fats suggests $\beta$-oxidation, especially by analogy with the oxidation of straight chain compounds *in vivo*. $\beta$-Oxidation would explain, also, the production of methyl ketones in essential oils (*e.g.* methylnonyl ketone in oil of rue), and the occurrence of ketones in the "échappées" of coconut oil. Haller and Lassieur [1910] found

[1] (This paper is based on a Thesis approved for the Degree of Doctor of Philosophy in the University of London, June, 1925.)

that the "échappées," or oily substance which collects on the surface of the condensed steam in the deodorising of coconut oil, consists of methylheptyl, methylnonyl and methylundecyl ketones with about 12 % methylheptyl and methylnonyl carbinols. These ketones, to which the disagreeable odour and flavour of the crude, acid-free oil is due, must be formed during the drying of the copra, since coconut oil expressed from the fresh fruit has an agreeable almond-like odour and taste. Their formation may be ascribed either, as Haller and Lassieur suggest, to the decomposition under enzymic action of some hitherto unknown constituent of the coconut or, in the light of the present work, to the action of moulds, which during the sun-drying of the copra develop on it, splitting and oxidising the fat to methyl ketones. Much of the dry copra arriving from abroad is certainly infected with mould spores.

It will be noted that Haller and Lassieur found methyl ketones corresponding to three only of the known fatty acids of coconut oil.

It is possible that moulds cannot assimilate the higher fatty acids; moreover, certain fatty acids act as poisons even in weak concentration. *Penicillium glaucum* grows in a concentration of 24 mg. of caproic acid per 100 cc., but not in the same concentration of caprylic acid [Laxa, 1902]. Spieckermann [1912] states that it can grow in comparatively strong acetic acid but not in weak propionic acid, and concludes that the poisoning capacity of the acids increases with molecular weight up to caprylic acid, and then diminishes. Stärkle [1924] suggests that the poisonous acids are neutralised, as they are formed, by ammonia produced by the growing organism, and this is supported by the contention of Jensen [1902] that ammonium salts of fatty acids are produced by mould action on butter. Accordingiy Stärkle gives the course of the oxidation as follows:

$$R.CH_2.CH_2.COONH_4 \rightarrow R.CH(OH).CH_3 \rightarrow R.CO.CH_3.$$

Dakin, however, obtained ketones even when caustic soda was used as neutralising agent. Stärkle does not explain the anomalous action of *Oidium lactis*, which, though rich in hydrolysing and oxidising enzymes, is unable to produce methyl ketones.

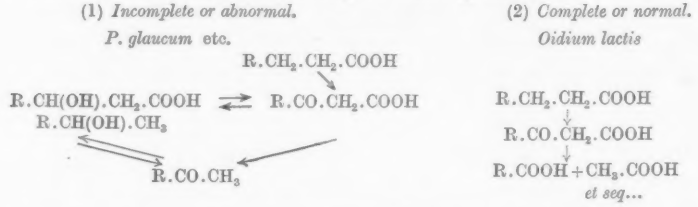
The secondary alcohols detected by Haller and Lassieur correspond to two of the ketones, and might therefore appear to be intermediate products in the oxidation. On the other hand they may be formed by reduction of the ketones. Thus, dextrorotatory methyl carbinols are produced by the action of yeasts on the corresponding ketones [Neuberg and Nord, 1919], but Dakin [1910] did not detect $\beta$-hydroxy-acids in his fatty acid oxidations. Again, in actions *in vitro* $\beta$-hydroxy-acids do not split off $CO_2$ as keto-acids readily do; while in oxidations *in vivo* the fatty acids are oxidised directly to the keto-acids, the $\beta$-hydroxyacetic acid of diabetes being probably a secondary reduction product of acetoacetic acid.

While agreeing with Stärkle that mould oxidation is analogous to Dakin's synthesis, the author believes that owing to the poisonous or narcotic effects of

volatile fatty acids on moulds, the oxidation is really abnormal. Under such unfavourable conditions the normal life processes are restrained and respiration is impeded. When the mould grows on a medium of low nutritive value, ketones are more readily formed, as is confirmed by Derx [1925].

*Oidium lactis*, on the other hand, offers strong resistance to external influences [Lang and Freudenreich, 1893] and can even destroy living yeast cells. Probably, therefore, it can effect a more complete or normal decomposition of fatty acids than can *Penicillium* or *Aspergillus*.

The course of the oxidations may be thus represented:




The normal form is supported by the fact that in butter *O. lactis* produces volatile acids [Jensen, 1902]. The oxidation of the non-poisonous acids, even by *Penicillium*, probably follows scheme (2), but oxidation of poisonous acids stops short at the keto-acid, which then splits up to methyl ketone. *O. lactis*, on the other hand, effects acid hydrolysis of the keto-acid to a lower acid, which in turn may be further oxidised.

The theory outlined above has formed the basis of the experimental work. This includes (I) an investigation of the products of the action of a *Penicillium* organism on coconut oil, (II) physiological experiments with *Penicillium* and *O. lactis*, (III) a repetition of Dakin's oxidation of fatty acids to determine, if possible, whether secondary alcohols are formed as intermediate products, and (IV) a more complete investigation of the "échappées" of Haller and Lassieur.

## Experimental.

### I. The action of *Penicillium* on coconut oil.

A pure culture was prepared of a mould organism isolated from rancid coconut oil margarine. The organism was identified as *Penicillium palitans* Westling [Westling, 1912], this being kindly checked by Dr Derx of Delft, Holland, to whom the author wishes to acknowledge his thanks.

*Preparation of rancid coconut oil and separation of the rancidity products.*

The oil was the refined deodorised oil used in margarine manufacture and was taken from a consignment from Oliefabriek Calvé-Delft, Delft. 20 cc. of just melted fat was emulsified with 20 cc. Raulin's solution containing 10 % gelatin, previously sown with spores from the pure culture, and the resulting emulsion poured into a 6-inch Petri dish placed on a cold slab. When the

emulsion had set the plates (80 in each weekly batch) were placed in an incubator at 20°. At the end of each week, when the plates showed a luxuriant growth and gave the characteristic odour, they were warmed, the melted contents poured into a 5-litre distillation flask and distilled from an oil-bath in a current of nitrogen. The delivery tube of the flask was connected by two double surface condensers to a conical receiver with a side outlet attached to a U-tube containing chloroform. This tube stood in powdered ice, its purpose being to trap any volatile matters carried away by the gas. The temperature of the bath was raised gradually to 150° and maintained at this until the water in the flask had wholly passed over. At this point the odoriferous substances had apparently completely distilled, and an oil was observed floating on the surface of the aqueous distillate. The latter was collected and preserved in tightly stoppered bottles in the dark; the fatty residue in the flask was also kept. At the end of this part of the work, 43 kg. of fat had been treated, 40 litres of aqueous distillate collected, and 4·7 kg. of mould grown. The distillate was treated five times with pure chloroform to extract the rancidity products, yielding eventually 100·5 g. crude aromatic oil.

*Examination of odoriferous rancidity products.*

These might be a mixture of ketones, aldehydes, esters, secondary alcohols, free fatty acid and neutral fat. Separation was accomplished as follows.

(A test for aldehydes with Fehling's solution showed only the merest trace.)

(*a*) *Free acid.* The liquid was washed with 10 % $Na_2CO_3$ solution, then with water. Only a small amount of acid was obtained.

(*b*) *Neutral fat.* After drying over anhydrous sodium sulphate the liquid was distilled, first at ordinary pressure, then *in vacuo*. The residue proved to be neutral fat, Wt. 5·48 g.

(*c*) *Alcohols.* These were separated by Haller's method [Haller and Lassieur, 1910] of conversion to the phthalic acid esters, followed by extraction with 10 % $Na_2CO_3$, acidification and saponification. The alcohols had an odour resembling that of the ketonic portion, Wt. 3·3 g.

(*d*) *Esters.* The remaining liquid was saponified with methyl alcoholic potash. The alcohols were obtained from the unsaponifiable matter by Haller's method, as above, and the acids from the soap solution,

Weight of ester alcohols 0·30 g.
Weight of ester acids 0·93 g.

(*e*) *Ketones.* These constituted the remainder of the unsaponifiable matter of (*d*). Wt. (dried) 71·0 g.

As the ketones constituted the main part of the rancidity products they were examined first.

1. *The ketones.* The liquid was dark coloured and had a strong odour of rancid coconut oil: $D_{4°}^{15·5°}$ 0·826; it reacted with saturated sodium bisulphite solution but did not reduce Fehling's solution. After six distillations, in which a Young

"8 pear" fractionating column was used, the liquid was resolved into the following fractions:

Table I. *Wt. of substance distilled* 71·0 g.

| Fraction | Temperature °C | Weight g. | |
|---|---|---|---|
| 1 | 150 | 1·40 | |
| 2 | 150–152 | 24·44 | refractionated |
| 3 | 152–157 | 4·10 | 150–2 2·45 |
| 4 | 157–168 | 1·17 | 152–7 1·65 |
| 5 | 168–173 | 1·40 | |
| 6 | 173–188 | 1·62 | |
| 7 | 188–196 | 8·49 | |
| 8 | 196–205 | 2·47 | |
| 9 | 205–215 | 2·64 | |
| 10 | 215–223 | 2·71 | |
| 11 | 223–228 | 9·64 | |
| 12 | residue | 5·54 | |
| | Total | 65·62 | Loss 5·38 g. |

The residue was dark coloured and viscous, with a slight odour of acrolein, due probably to some decomposition during the distillation. All fractions had the strong, pungent odour of the original rancid oil, which, however, decreased in intensity with rise of the boiling-point. All reacted with an alcoholic solution of semicarbazide hydrochloride to form semicarbazones, and also with *p*-nitrophenylhydrazine.

*Fraction* (2) B.P. 150–152°. On refractionation the bulk of the liquid distilled at 151–152°. This fraction contained 12·20 % H and 73·63 % C; calculated for $C_7H_{14}O$, 12·28 % H, 73·68 % C.

The semicarbazone was obtained in colourless crystals, soluble in alcohol; M.P. 122·5–123°; M.P. of the semicarbazone of synthetic methyl-*n*-amyl ketone 122°; mixed M.P. 122°.

Fraction (2) was therefore methyl-*n*-amyl ketone.

*Fraction* (7) B.P. 188–196°. On refractionation the bulk of the liquid distilled at 193–195°. This fraction contained 12·63 % H and 75·89 % C; calculated for $C_9H_{18}O$, 12·67 % H and 76·06 % C.

M.P. of the semicarbazone 118°; M.P. of the semicarbazone of synthetic methylheptyl ketone 118°; mixed M.P. 118°.

Fraction (7) was therefore methylheptyl ketone.

*Fraction* (11) B.P. 223–228°. On refractionation the portion distilling at 224–226°, the B.P. of methylnonyl ketone, was collected. This fraction contained 12·93 % H and 77·74 % C; calculated for $C_{11}H_{22}O$, 12·94 % H, 77·64 %C.

M.P. of the semicarbazone 122–122·5°; M.P. of the semicarbazone of synthetic methylnonyl ketone 123°; mixed M.P. 122°.

Fraction (11) was therefore methylnonyl ketone.

*Fractions* (3)–(6); (8)–(10). Semicarbazones were prepared from each fraction and, in most cases, pure substances were obtained after from 5 to 13 crystallisations from alcohol. No new substance was found, this indicating that the fractions were mixtures of the ketones already identified.

*Fraction* (1) B.P. up to 150°. The semicarbazone was found to be that of methylamyl ketone. Methylpropyl ketone could not be identified.

*Fraction* (12). The liquid was not wholly soluble in cold alcohol. The insoluble oil being separated, the clear alcoholic solution was used to prepare the semicarbazone, which proved to be that of methylnonyl ketone. The semicarbazone of methylundecyl ketone could not be found.

2. *Neutral fat.* Saponification value 246·8, iodine value 13·7; the fat was completely saponifiable. It therefore closely resembled coconut oil.

3. *Alcohols.* The yield of alcohols being only about 4 cc., it was decided to examine the products formed on oxidation. On distillation from a very small flask, the liquid was resolved into two fractions, (1) 160–200°, (2) 200–240°, and a drop of dark residue. The fractions were oxidised with chromic acid mixture as follows. 2 g. finely powdered potassium dichromate were added to each liquid, contained in a small flask surrounded by crushed ice. A mixture of 3 g. $H_2SO_4$ and 4 g. water was allowed to drop slowly from a tap funnel into each flask, so that the whole of the acid was added in 12 hours. After dilution with water, the reaction products were extracted with ether, washed free from acid and dried. On evaporation of the ether, 2 cc. of a pungent smelling liquid was in each case obtained. With Fehling's solution there was no reaction, but with *p*-nitrophenylhydrazine each liquid gave a positive reaction for ketones.

The alcohols, therefore, consisted of secondary alcohols.

The ketones were now converted to their semicarbazones.

*Semicarbazones* (crystallised from alcohol):

(i) Alcohol fraction B.P. 160–200°

| | 1st crop | 2nd crop |
|---|---|---|
| | M.P. | M.P. |
| 5th crystallisation | 117–118° | 117–118° |
| 6th ,, | 118–118·5° | 120–121° |
| 7th ,, | — | 121–122° |

(ii) Alcohol fraction B.P. 200–240°.

| | 1st crop | 2nd crop |
|---|---|---|
| | M.P. | M.P. |
| 6th crystallisation | 119–120° | not sufficient for determination |
| 7th ,, | 121° | |
| 8th ,, | 122–123° | |

(i) *Semicarbazone from 1st crop* (*6th crystallisation*) M.P. 118–118·5°.

Analysis: 10·65 % H, 60·71 % C; calculated for $C_{10}H_{21}ON_3$, 10·55 % H, 60·30 % C; mixed M.P. with semicarbazone of synthetic methylheptyl ketone 117–118°.

Hence alcohol fraction (i) contained methylheptyl carbinol.

(i) *Semicarbazone from 2nd crop* (*7th crystallisation*) M.P. 121–122°.

Mixed M.P. with semicarbazone of methylamyl ketone 120–121°.

Hence alcohol fraction (i) contained methylamyl carbinol. (Analysis on 0·023 g. gave an unsatisfactory result.)

(ii) *Semicarbazone from 1st crop* (*8th crystallisation*) M.P. 122–123°.

Analysis: 11·64 % H, 63·10 % C; calculated for $C_{12}H_{25}ON_3$, 11·01 % H, 63·43 % C; mixed M.P. with semicarbazone of methylnonyl ketone 121–122°.

Hence alcohol fraction (ii) contained methylnonyl carbinol.

4. *Ester acids and ester alcohols.*

(i) *Acids.* Neutralisation value, 382·7; mean molecular weight, 146·6. Calculated for caprylic acid M.wt. 144.

The barium soap was prepared and crystallised from hot water. 1st crop of crystals, Ba 32·30 %; 2nd crop of crystals, Ba 32·65 %; required for barium caprylate, Ba $(C_8H_{15}O_2)_2$, Ba 32·45 %.

The esters were, therefore, principally those of caprylic acid.

(ii) *Alcohols.* Owing to the possibility of ethyl esters being present in the rancidity products, the acid phthalic esters were saponified with methyl alcoholic potash (ester alcohol separation). This precaution was justified, for the quantity of alcohols insoluble in water (0·3 g.) was too small to account for the ester acids found. The aqueous liquid containing methyl alcohol and any soluble alcohols was neutralised and submitted to several successive distillations until a concentrated alcoholic distillate was obtained. Ethyl alcohol was identified in this distillate by the iodoform reaction and by oxidation to acetaldehyde.

The alcohols insoluble in water yielded, on oxidation with chromic acid mixture, a small quantity of ketones. The semicarbazone was prepared, but the quantity was insufficient for purification. The secondary alcohols were probably identical with those present in the free state.

The discovery of ethyl esters suggested the possible presence of free ethyl alcohol, but the method of investigation had precluded its detection. Accordingly, a further 1600 cc. of rancid coconut oil was distilled, and, the ketonic oil being separated, the aqueous distillate at length yielded a concentrated alcoholic fraction. Ethyl alcohol was here definitely identified. It was probably produced by fermentation of the sugar in the medium.

5. *Free acid.* This was added to the fatty acids obtained from the aqueous part of the distillate.

*Examination of aqueous part of distillate.*

The liquid was made alkaline with sodium hydroxide and evaporated to small bulk. Ammonia was liberated in considerable quantity, showing the presence of ammonium salts. The barium soaps of the fatty acids were prepared and fractionally crystallised from hot water, and by their analysis lauric, capric and caprylic acids were identified. Caproic acid was probably also present, as a final crystallisation product gave a mean molecular weight of 140·8 (caprylic acid M.wt. 144). The lower fatty acids, including acetic acid could not be detected.

*Examination of the coconut oil from which the volatile rancidity products had been removed.*

After the determination of the analytical characteristics of the fat, the free fatty acids were separated and converted to their methyl esters, which were

then fractionally distilled *in vacuo*. The original coconut oil was analysed in the same way.

Table II.

| | Fresh oil | Rancid oil | Rancid oil after distillation | The fatty acids separated from rancid oil |
|---|---|---|---|---|
| Sapon. value | 258·9 | 259·7 | 258·0 | 264·5 |
| Iodine value | 8·0 | 8·55 | 8·4 | 15·6 |
| Reichert Meissl | 7·54 | 7·81 | 7·7 | 5·39 |
| Polenske | 16·35 | 16·70 | 14·95 | 11·35 |
| Acetyl No. | 1·2 | 7·7 | 6·2 | — |

Composition of the original coconut oil and of the free acids separated from the rancid fat (calculated from fractional distillation of methyl esters):

Table III.

| | Original oil % | Separated free acids % |
|---|---|---|
| Caproic acid | 0·2 | — |
| Caprylic acid | 7·2 | 0·2 |
| Capric acid | 10·7 | 10·4 |
| Lauric acid | 48·7 | 42·5 |
| Myristic acid | 17·5 | 23·9 |
| Palmitic acid | 5·4 | 4·5 |
| Stearic acid | 0·8 | 1·4 |
| Oleic acid | 9·0 | 16·6 |
| | 99·5 | 99·5 |

The analyses were carried out under the same conditions, but, as the calculations were based on the assumption that each fraction consisted of not more than two esters and oleic ester, the results are useful only for comparison.

The most striking feature of the results is the decreased percentage of caprylic acid, with the corresponding increase of the higher acids. Caprylic acid, therefore, is apparently almost completely consumed by the mould, while the higher acids are attacked either to a less extent or not at all. This is confirmed by the analysis of the ketones from the rancid fat, where methylamyl ketone was found to predominate.

If the glycerides of the various fatty acids of coconut oil are split equally by mould lipase (and this is the view of several observers [Jensen, 1902]) it follows that acids higher than lauric acid are not oxidised to ketones. This is confirmed by the analysis of the ketones. It is possible that the higher fatty acids, if absorbed, are partially decomposed according to the complete oxida tion scheme suggested for *Oidium lactis*.

## II. Physiological experiments with *Penicillium palitans* and *Oidium lactis*.

To obtain further information of the selective action of moulds on fatty acids and light on the anomalous behaviour of *Oidium lactis*, various series of experiments were carried out with both organisms under the same conditions. Pure cultures were grown on gelatin—Raulin's solution, containing graduated

proportions of pure fatty acids, secondary alcohols, methyl ketones and keto-esters (prepared by the acetoacetic ester synthesis). The products formed after seven days' incubation at 20° were obtained by steam-distilling the cultures and extracting the aqueous distillate with ether and were examined. Ketones were tested for by means of the *p*-nitrophenylhydrazine or the semicarbazide reaction.

(*a*) *Action of the moulds on pure fatty acids.*

Table IV.

| | *P. palitans* | | *O. lactis* | |
|---|---|---|---|---|
| Acid | Quantity inhibiting growth % | Ketone formation | Quantity inhibiting growth % | Ketone formation |
| Caproic | >0·10 | + | >0·04 | – |
| Caprylic | >0·04 | + | no growth at 0·025 | – |
| Capric | >0·8 | + | >0·20 | – |
| Lauric | >25 | + | >25 | – |
| Myristic | >25 | ? | >25 | – |
| Palmitic | >25 | – | >25 | – |
| Stearic | >25 | – | >25 | – |
| Butyric | >0·04 | – | >0·5 | – |

In the experiments with *Penicillium*, in which growth took place, the mould remained white, with little conidia formation, the mycelium and hyphae tending to grow into the air. The results support the earlier conclusion, that only acids up to lauric acid are oxidised to ketones. The poisonous character of the acids was clearly demonstrated. Caprylic acid exerted the greatest effect; the higher acids affected the organism very little, if at all, this being due probably to their non-absorption on the mycelium.

The results for *O. lactis* confirm the author's previous work. It would seem that the lower fatty acids are more poisonous to *O. lactis* than to *Penicillium*. The case of butyric acid, where the order is reversed, is noteworthy, and it may be that, its normal habitat being milk and butter, *O. lactis* has acquired tolerance towards butyric acid.

(*b*) *Action of the moulds on secondary alcohols.*

Table V.

| | *P. palitans* | | *O. lactis* | |
|---|---|---|---|---|
| Secondary alcohol | Quantity inhibiting growth % | Ketone formation | Quantity inhibiting growth % | Ketone formation |
| Methylamyl | >0·8 | + semicarbazone M.P. 122° | 0·1 | – |
| Methylheptyl | >2·0 | + semicarbazone M.P. 117° | 0·2 | – |
| Methylnonyl | >4·0 | + semicarbazone M.P. 121° | 2·0 | – |

The results support the idea that secondary alcohols may be intermediate products in the oxidation of fatty acids by moulds of the *Penicillium* genus.

*P. palitans* exerts a powerful oxidising action on the alcohols, which exhibit little poisonous action. *O. lactis*, on the other hand, suffers a marked poisonous effect, and oxidation, where it occurs, is presumably more complete.

(*c*) *Action of the moulds on methyl ketones.*

Table VI.

| Ketone | *P. palitans* Quantity inhibiting growth % | *O. lactis* Quantity inhibiting growth % |
|---|---|---|
| Methylamyl | >0·2 | >0·1 |
| Methylheptyl | >0·8 | >0·1 |
| Methylnonyl | >2·0 | >0·2 |

The two organisms show a great difference in their behaviour. *Penicillium* can withstand considerable quantities of methyl ketones, which it partially destroys; *Oidium* very little.

(*d*) *Action of the moulds on keto-esters.*

The cultures after incubation were examined for ketones and acidity. Although the acidity figures were checked against blank experiments, they cannot be taken as reliable, as more or less acid may be consumed by the mould, or neutralised by formation of ammonium compounds.

Table VII.

| Keto-ester | *P. palitans* Quantity inhibiting growth % | *P. palitans* Ketone formation | *P. palitans* Acidity *N*/10 NaOH cc. | *O. lactis* Quantity inhibiting growth % | *O. lactis* Ketone formation | *O. lactis* Acidity *N*/10 NaOH cc. |
|---|---|---|---|---|---|---|
| Acetoacetic ester | >0·2 | – | 3·0 | >0·4 | – | 0·4 |
| Butylacetoacetic ester | >0·4 | – | 2·0 | >0·4 | – | 0·6 |
| Amylacetoacetic ester | >1 | – | 1·9 | >2·0 | – | 4·1 |

Since in none of the cultures were ketones detected, while the production of acid was evident, it would seem that both organisms decompose the keto-esters by acid hydrolysis. The lower acidity figures and the negative result for *O. lactis* must be explained by its greater capacity for producing ammonia. Keto-esters (or perhaps the fatty acids produced by their decomposition) are poisonous to the organisms. This was confirmed by the appearance of the cultures; growth, at first vigorous, became more and more feeble, both organisms tending to throw out aerial hyphae. In the *Penicillium* experiments conidia formation was observed only in the cultures with the smallest ester concentration; in the other cultures the mycelium remained white.

A similar set of experiments, with sterilised milk as medium, also showed acid formation by both organisms; especially by *O. lactis*, which in some cases produced an acidity equivalent to 20 cc. *N*/10 NaOH in the culture.

The normal process of decomposition of keto-esters (keto-acids) is apparently the same for both organisms; ketonic fission must be abnormal.

Further experiments were conducted with 25 % beef-fat emulsified with the medium. Beef-fat, consisting essentially of the glycerides of palmitic, stearic and oleic acids, does not yield ketones under mould action; this was confirmed by the blank experiments, where no keto-esters were used.

*Action of the moulds on keto-esters in presence of* 25 % *beef-fat.*

Table VIII.

| | *P. palitans* | | *O. lactis* | |
|---|---|---|---|---|
| Keto-ester | Quantity inhibiting growth % | Ketone formation | Quantity inhibiting growth % | Ketone formation |
| Blank | — | – | — | – |
| Acetoacetic ester | 0·4 | + | 0·4 | – |
| Butylacetoacetic ester | 2·0 | + | 2·0 | – |
| Amylacetoacetic ester | 2·0 | + | 2·0 | – |

Identical results were obtained, using palmitic acid in place of beef-fat.

The positive reactions for ketones were quite distinct but the quantities produced were evidently small. The reason is probably that the beef fatty acids, formed by the original hydrolysis, have a lesser clogging effect on the mycelium than the volatile acids.

The experiments show that with *Penicillium*, but not with *Oidium*, a medium containing fat or fatty acid, and therefore hindering respiration, causes ketonic decomposition of the keto-ester. This explains the difference in the behaviour of the organisms towards fats. The fatty acids (up to lauric acid) formed by hydrolysis are absorbed or adsorbed on the mycelium of the organism and, being poisonous, are oxidised by excited oxidase secretion to keto-acids, which tend to split up to methyl ketones. The ketones are still deleterious to the *Penicillium*, though to a lesser extent than the fatty acids. To *Oidium*, on the other hand, they are specially harmful; but, possessing more powerful enzymic properties, *Oidium* is able to restrain the tendency of the keto-acids to split off $CO_2$ and to hydrolyse them to lower fatty acids and acetic acid, which are less harmful than ketones. Acetic acid is utilised by the organism; thus the fat is gradually decomposed and consumed.

This theory, though not without fault, seems to be in best accord with the experimental data. It is in agreement moreover, with the properties of *O. lactis* in its natural habitat, milk. The organism prefers an acid medium, the acid being of low carbon content (lactic acid), and in butter-fat finds a fat admirably suited to its peculiarities; the highly poisonous acids, caproic and caprylic, are present in small quantity, while butyric acid is, as experiment showed, readily utilised as a source of energy. *Penicillium*, as it would be expected, produces only traces of ketones in butter-fat.

### III. The Dakin synthesis with special reference to secondary alcohols as intermediate products.

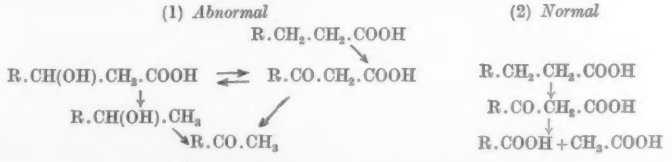
100 g. of the fatty acids of coconut oil were neutralised with ammonia and a slight excess of ammonia added. After boiling with a large excess of hydrogen peroxide for 12 hours under a reflux condenser, the mixture was distilled. The ketonic products were extracted from the distillate with ether and, after treatment by Haller's method for the separation of alcohols, yielded 5–6 g. ketones and 0·5 g. of a dark-coloured oil. Oxidation of this oil in the cold by chromic acid mixture produced a few drops of liquid which reacted with semicarbazide hydrochloride.

Further experiments were conducted with (*a*) 30 g. caprylic acid, (*b*) 41 g. capric acid and (*c*) 220 g. lauric acid. In (*a*) and (*b*) only a few drops of alcohols were obtained; in (*c*) however 0·33 g. resulted, giving 0·108 g. on oxidation. In all experiments the presence of ketone was detected in the products of oxidation of the alcohols, and in (*c*) the semicarbazone was prepared and crystallised from alcohol: 2nd crystallisation, M.P. 121–122°; semicarbazone of methylnonyl ketone, M.P. 122–123°; mixed M.P. 120°.

The ketone in (*c*) was therefore methylnonyl ketone corresponding to methylnonyl carbinol.

The ketones, from which the alcohols had been separated, were identified as methylamyl, methylheptyl and methylnonyl respectively, thus confirming Dakin's work.

The results appear to indicate that secondary alcohols are intermediate products in the oxidation of fatty acids, but the evidence is not convincing. The identification of secondary alcohols by oxidation to the same ketones from which they were originally separated is certainly a weak point; the yield, however, was too small for such a test as the pseudo-nitrol reaction. Again, the fact that *Penicillium* oxidises secondary alcohols to ketones cannot be taken as proof that they are intermediate products. But if not intermediate products, they must be produced by reduction of ketones; that is, the mould must, at a later stage, probably when narcotised by the poison, reduce, not oxidise. Possibly it seeks for a fresh source of oxygen, by freeing a reducing substance (hydrogen?) which reacts with the ketone. In this case it would be expected that a pinacone would also be formed, but this, though specially looked for, could not be found. In the absence of further evidence, therefore, and on the data of the foregoing work, the author explains the course of the oxidation of fatty acids by moulds thus:



### The "échappées" of Haller and Lassieur.

The work of Haller and Lassieur was repeated and extended, substances being found additional to those recorded by Haller and Lassieur, and it is proposed, therefore, to make this work the subject of a separate paper.

### Summary.

1. The rancidity of coconut oil, caused by a typical *Penicillium* organism, is due essentially to the presence of methylamyl, methylheptyl and methylnonyl ketones. Methylamyl ketone occurs in the greatest quantity and is responsible for the characteristic "perfume" odour of the rancid oil. There are also present secondary alcohols corresponding to the ketones, ethyl alcohol (probably by fermentation of sugar), esters of the secondary alcohols and ethyl alcohol with caprylic acid (and probably other fatty acids), and free fatty acids.

2. The production of methyl ketones by moulds shows that here, as in the higher animals and man, oxidation of a chain compound takes place primarily at the $\beta$-carbon atom, with formation of a keto-acid.

3. The normal course of the decomposition of the keto-acid by moulds is the formation of a fatty acid containing two carbon atoms less, and acetic acid; but in the case of *Penicillium* the absorption of poisonous fatty acids on the mycelium impedes respiration, and, an abnormal condition being induced, the keto-acid is decomposed to methyl ketone and carbon dioxide.

4. The poisoning capacity of the fatty acids towards *Penicillium* increases with the molecular weight up to caprylic acid, then decreases. Only acids up to lauric acid are absorbed; consequently, ketones of higher molecular weight than methylnonyl ketone are not formed.

5. The fatty acids with the exception of butyric acid and perhaps the lower acids, are more poisonous to *O. lactis* than to *Penicillium*. *Oidium*, nevertheless, does not induce ketonic fission of the keto-acid, its greater enzymic activity making possible the normal decomposition to a lower acid and acetic acid.

6. Secondary alcohols are probably intermediate products of the mould oxidation, but the experimental evidence is not yet conclusive.

7. Dakin's oxidation of saturated fatty acids has been repeated, but the formation of secondary alcohols as intermediate products could not be satisfactorily demonstrated.

In conclusion the author wishes to express his indebtedness to Mr G. Hope, B.Sc., for his assistance in the analyses and in other parts of the work.

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# XV. VASODILATOR CONSTITUENTS OF TISSUE EXTRACTS.

## ISOLATION OF HISTAMINE FROM MUSCLE.

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In a previous communication [Best, Dale, Dudley and Thorpe, 1927] we were able to prove that the chief vasodilator constituent of both ox liver and lung was histamine ($\beta$-iminazolylethylamine) by the isolation of this substance in a state of chemical purity from alcoholic extracts of each organ under conditions which precluded its formation by autolysis or bacterial action during the process of isolation.

Now since extracts of almost all organs of the body have been shown to provide depressor substances, it would be of considerable physiological importance if more definite information as to the chemical nature of these could be obtained; for until our own investigations began no depressor substance other than choline had been isolated under conditions which are absolutely free from suspicion of contamination by bacterial action. The important work of Abel and Kubota in this connection has been considered in the paper quoted above. With a view to selecting the most suitable organ for examination it became necessary to review the distribution of the depressor substances; for although this field has been explored by a number of workers their extracts have been both prepared and tested by different methods. To collect such data and use them as a comparative survey of the distribution of the vasodilators would in all probability be very misleading. Thus it seemed that reliable information could only be obtained by preparing and estimating the activity of the extracts under standardised conditions.

For this purpose the alcohol method of extraction already described [Best, Dale, Dudley and Thorpe, 1927] seemed preferable since, while absolute sterility was ensured, this method would be least likely to permit of any hydrolytic changes taking place.

Extracts from a number of organs of horse and ox have been prepared by the alcohol method and the extracts thus obtained, after removal of alcohol and fat, were tested on the blood-pressure of the cat in comparison with pure histamine. Except in the case of spleen no attempt has been made to determine the influence of choline on the histamine-like activity. Since the values

were only intended as a guide for the selection of material for future chemical work, the additional labour of estimating the histamine and choline separately did not seem to be justified, for the depressor activity of 1 mg. of histamine is only matched by quantities of choline varying from 300 mg. to 3000 mg. according to the sensitiveness of the cat employed.

All the organs mentioned in Table I were worked up within an hour of the killing of the animal. In most cases the experiments are in duplicate and values from two different animals are given. For convenience in comparison, the values obtained for liver and lung, already published, are included. It is hardly necessary to point out that in the large scale experiments any slight error in the matching of the activity with that of histamine (the normal dose of histamine was of the order of 0·003 mg.) would be very greatly magnified in calculating the total activity of the extract. On the other hand the losses involved in making extracts of small organs weighing only a few grams would be proportionately much greater than those involved in preparing an extract from 500 g. or more material.

Table I. *Distribution of depressor substances in various organs.*

| Organ | Depressor activity expressed as mg. histamine per kg. fresh tissue | Source | Remarks |
|---|---|---|---|
| Lung | 44, 75 | Ox | 75 = L.S. |
| ,, | 35 | Horse | — |
| Ovary | 9 | Ox | K. |
| Plain muscle (bladder) | 7, 8 | Horse | — |
| Spleen | 7·5, 5 | ,, | Values from atropinised cat. C. (Values before atropine 9 and 17) |
| Parotid gland | 5·3, 6·7 | ,, | c. |
| Liver | 5·4, 5·0 | Ox | L.S. } C. |
| ,, | 2·5, 6·6 | Horse | — } |
| Kidney | 2·6, 3·3 | ,, | — |
| Pancreas | 1·6, 3·0 | ,, | c. |
| Testis | 1·8 | Ox | K. |
| Striated muscle | 1·0 | Cat | — |
| ,, | 1·0 | Dog | After purification with basic lead acetate |
| ,, | 1·1, 1·4 | Horse | — |
| ,, | 4·0 | Ox | L.S. |
| Submaxillary gland | 0·6 | Horse | — |
| ,, | 0·5 | ,, | — |
| Thyroid gland | 0·5 | ,, | K. |
| Suprarenal gland | 0·3 | ,, | Purified with phosphotungstic acid to remove adrenaline |

L.S. = Values obtained from extracts prepared on a large scale.
C. = Choline present in large quantity.
c. = Physiological tests suggested presence of choline.
K. = Extracts had to be kept some time before testing—values probably low.

Considering the nature of the experiments the above results show good agreement. The most serious discrepancies are seen with liver extracts. Here choline is known to be present in such large quantities that the depressor activity is greater than that which would be produced by the summation of the

separate effects of the histamine and choline actually present [Best, Dale, Dudley and Thorpe, 1927, p. 403]. Hence the low value (2·5), obtained with a cat not very sensitive to choline, is considered to be more nearly the correct value for the histamine content.

The unique position of lung extracts in this list has already been discussed [Best, Dale, Dudley and Thorpe, 1927] and requires no further comment here.

The relatively high activity of spleen extract makes this organ suitable for examination. On physiological grounds also an examination of spleen would be interesting in view of the work of Stern and Rothlin [1919] and others. A preliminary experiment with horse spleen suggested that histamine was probably present, the active substance being concentrated with little loss into the fraction from which histamine was actually isolated in the experiments with lung. The presence of choline in spleen was confirmed.

At the same time a preliminary experiment was carried out with striated muscle, for, although the vasodilator activity of this tissue was relatively low, it was of considerable physiological importance to have decisive information as to the chemical nature of the substance or substances responsible for this activity. This communication, therefore, is mainly concerned with the isolation of a vasodilator substance from muscle which has been identified as histamine.

### The isolation of histamine from striated muscle.

Preliminary experiments made it obvious that the isolation of histamine from muscle extracts would be a difficult technical task. The activity of the extracts was poor, corresponding to only about 2 mg. histamine per kg. of fresh meat, and the extracts were much more complicated than those of liver and lung with respect to basic extractives. The amount of accompanying material was so great that only a small final yield of histamine could be expected.

In a preliminary exploratory experiment the procedure successfully employed for lung was observed. The activity was concentrated into the histidine-arginine fraction with little loss. When, however, the mass was dried with baryta only 14 % of the activity could be recovered by repeated extraction with alcohol. This was found to be due to the presence of large quantities of substances of an amino-acid or simple peptide nature. When the alcoholic extract was examined it was found to consist largely of methylguanidine (and small amounts of creatinine) which was precipitated, along with the active substance, by picric acid. The relative quantities of the two substances (2 g. methylguanidine to 3 mg. histamine picrate) made it obvious that no hope of separating the active substance could be entertained.

Two problems thus arose: (1) the removal of methylguanidine, and (2) the reduction of the quantities of amino-acid-like substances in the mass to be extracted with alcohol.

(1) It is now generally accepted that methylguanidine does not exist as such in fresh muscle, and that in those cases where it has been isolated it has

been formed from creatine by the action of the silver oxide during the silver fractionation. The methylguanidine actually found only represented a small part of the creatine originally present, for large quantities of the latter crystallised out at an early stage. An attempt to remove the creatine remaining (before proceeding to the silver fractionation) in the form of potassium creatinine picrate proved to be impracticable because the picrate carried down 50 % of the activity.

According to the literature methylguanidine comes down in the arginine and creatinine in the histidine fractions of the Kossel-Kutscher silver fractionation. When, however, the histidine and arginine fractions of the muscle extract were separated it was found that the reverse was the case, the greater part of the methylguanidine coming down in the histidine fraction.

The problem was finally solved by a very simple modification of the original method. In order to reduce the amount of basic material accompanying the active substance, the phosphotungstates were extracted with acetone in which a small part was insoluble. On working up the acetone-soluble phosphotungstates alone (the active substance goes into this fraction with little loss) it was found that the histidine fraction was not only free from methylguanidine, although a small quantity of creatine was present, but also contained nearly the whole of the activity. The phosphotungstates which had been left after extraction with acetone consisted mainly of potassium phosphotungstate, with a small amount of organic base which has not yet been examined. It would seem that the anomalous results obtained in the silver fractionations in the exploratory experiments were probably due to the presence of large amounts of potassium salts.

(2) The same method also served to solve the second problem, for the histidine fraction was mainly composed of carnosine nitrate, most of which could be easily removed by allowing it to crystallise out from an alcoholic solution. In this way sufficient material was removed to make possible the isolation of histamine from the mother-liquor.

This mother-liquor, on drying with baryta and extracting with alcohol, gave a 33 % yield of the histamine. The alcoholic extract still contained a large amount of basic material, but fortunately only creatinine and histamine were thrown down by picric acid. By a long and difficult fractional crystallisation these two bases were finally partially separated and the vasodilator principle extracted from the fresh muscle was proved to be histamine.

## Experimental.

35 kg. perfectly fresh ox muscle were minced and stirred into 79 l. 95 % alcohol. After 24 hours the meat was removed in a basket centrifuge and re-extracted with 72 l. 60 % alcohol for a further 24 hours. The combined extracts, after addition of sulphuric acid (0·36 cc. 50 % acid per l.), which produced a slight precipitate, were filtered through paper pulp. The clear filtrate was concentrated *in vacuo* below 30° to about 4 l. This was heated up to 60°

in a water-bath and then left over-night in the cold room. In this way most of the fat collected as a solid cake on the top of the liquid and could be removed by filtration. At the same time 38 g. creatine, which had crystallised out, were also removed. The last traces of fat were extracted from the filtrate by shaking with ether. The fat-free aqueous solution was then concentrated *in vacuo* to 800 cc. and, after removing 42 g. creatine which had crystallised out, neutralised with sodium hydroxide and treated with basic lead acetate until no further precipitate was produced. The filtrate and washings from this precipitate, after removal of lead as sulphide, were concentrated *in vacuo* to a thin syrup (700 cc.).

50 % sulphuric acid was added to the solution to produce a concentration of 5 % of this acid. This caused the separation of 76 g. potassium sulphate, which was filtered off and washed with a little 5 % sulphuric acid. It was then practically free from organic matter. The filtrate and washings were treated with phosphotungstic acid (25 % in 5 % sulphuric acid) until precipitation was complete. 7·7 l. were required. The precipitate was filtered off, washed with 5 % sulphuric acid and sucked as dry as possible. Without further drying, the damp precipitate was stirred up with 4 l. 75 % acetone (freshly distilled). After standing over-night the brown supernatant liquid was syphoned off and the residue centrifuged. The residue was thrice re-extracted with acetone. The residue consisted largely of potassium phosphotungstate, but also contained some organic bases which will not be described in the present communication.

The combined acetone extracts were concentrated *in vacuo* and decomposed with baryta in the usual way, excess of baryta being removed as sulphate. The solution of the bases was concentrated to 1300 cc. and divided into four fractions by the Kossel-Kutscher method. 400 g. silver nitrate in 1 l. water were added and the resulting precipitate, the "purine" fraction, filtered off (83 g.). Hot saturated baryta was then added cautiously until a precipitate was no longer formed on testing with ammoniacal silver nitrate. The granular precipitate, the "histidine" fraction, was filtered off and washed. The filtrate was saturated with baryta to precipitate the "arginine" fraction. The mother-liquor from this forms the "lysine" fraction.

Only one of the four fractions, the histidine fraction, showed appreciable depressor activity. The purine, arginine and lysine fractions were practically inactive and will not be discussed further in this paper.

The histidine fraction was suspended in sufficient dilute sulphuric acid to render the liquid just acid to Congo red and treated with hydrogen sulphide. The filtrate from the silver sulphide and barium sulphate was neutralised with baryta, and after removal of barium sulphate evaporated *in vacuo* to a mass of almost colourless needles. The mass was dissolved in 600 cc. boiling 80 % alcohol and allowed to cool, when a crop (35 g.) of large well-formed needles separated. These were collected and the mother-liquor treated with 1 l. absolute alcohol, when a further 7·4 g. separated in smaller needles. The mother-

liquor from these was evaporated *in vacuo* to a crystalline mass and the previous process repeated, giving a further 3·3 g. needles.

In this way 45·7 g. needles, which proved to be carnosine nitrate[1], were removed from the histidine fraction with only slight loss of activity, the final mother-liquor containing 90 % of the depressor substance originally present in the histidine fraction. This mother-liquor was concentrated to a small volume and ground up with a large excess of barium hydroxide with the addition of a little plaster of Paris. This mass was thoroughly dried and ground to a fine powder which was extracted with absolute alcohol for 3 hours in a Soxhlet apparatus. The extract was diluted with water and neutralised with sulphuric acid which precipitated the small amount of baryta which had been extracted. After removal of the alcohol *in vacuo* the barium sulphate was filtered off. The filtrate was concentrated to a small volume when 0·72 g. of a relatively sparingly soluble crystalline substance separated. The mother-liquor was concentrated *in vacuo* to 6 cc. and treated with 30 cc. saturated picric acid. The crystalline precipitate (Crop 1) was collected and the mother-liquor treated with a further 90 cc., giving a second crop. The mother-liquor from Crop 2 was concentrated *in vacuo* to 40 cc. and 10 cc. picric acid added. On standing a crystalline precipitate (Crop 3) separated from the emulsion first formed. No further crystalline material could be separated from the mother-liquor, which was by now only feebly active.

The three crops weighed 0·85 g., 1·02 g. and 0·3 g. respectively, and melted (with decomposition) at 214°, 214°, and 202°. The first two, which appeared to be identical, were combined and recrystallised giving 1·67 g. needles which melted at 220° and were identified as creatinine picrate. The mother-liquors from the creatinine picrate and Crop 3 were subjected to a long and tedious fractional crystallisation. Since the separation of 44 different fractions was involved this process will not be described in detail. It will be sufficient to indicate the lines on which the fractionation was carried through.

In the early stages the course of the fractionation could not be guided by the melting points of the various crops, since it was only in the final stage that a picrate melting above 220° (the melting point of creatinine picrate) was obtained. Chief reliance had to be placed on experience of the appearances of the crystals under the microscope gained during the experiments with liver and lung extracts. The appearance of the crystals when a dilute solution of sodium hydroxide was allowed to flow up to them, when viewed through the microscope, was also of great value. The appearance of a fugitive bright red colour on the surface of the crystal indicated creatinine (Jaffé's reaction). A feeble reaction was given by impure histamine picrate crystals.

During the course of the fractionation mechanical separation of the crystals had to be resorted to at three stages, whilst at the end of the process a valuable separation was effected by removing a crop while crystallising from the warm solution. Crop (*a*), obtained from the warm solution, melted at

[1] The isolation of carnosine nitrate at this stage will be considered in a later communication.

215°, while Crop (*b*), from the cold solution, melted at 214°. On recrystallisation Crop *a* gave crystals melting at 233°, which on recrystallisation gave pure histamine picrate, whereas Crop (*b*) on recrystallisation gave creatinine picrate melting at 218°.

The final yield of histamine picrate, obtained in two fractions, was 16·5 mg. (= 3·3 mg. base). This had been obtained from 0·5 g. of picrate containing, according to physiological test, 10 mg. histamine. The final mother-liquors contained 3 mg. histamine. The loss of 3·7 mg. can reasonably be ascribed to material used for melting points and Jaffé reactions and to traces left on the creatinine separated during the fractionation.

A solution of 1·005 mg. of the histamine picrate from muscle in 10 cc. water had the same depressor activity on the cat's blood-pressure as a similar solution prepared from a known sample of pure histamine picrate. The Pauly reactions were quantitatively equal. The picrate from muscle, pure histamine picrate and a mixture of the two all melted together at 240° with decomposition.

### The yield of histamine from muscle.

The amount of active substance arriving at the end of each stage in the purification had been estimated on the cat's blood-pressure as in the experiments with liver and lung extracts. These values are given in Table II.

Table II.

| Stage | Activity in mg. histamine | |
|---|---|---|
| Crude extract | 140 | |
| After basic lead acetate | 140 | |
| Phosphotungstates {acetone-soluble | 58 | |
| Phosphotungstates {acetone-insoluble | — | 3·75 |
| Adsorbed on barium phosphotungstate | — | 8 |
| Not precipitated by phosphotungstic acid | — | 7 |
| Silver fractions {histidine | 42 | |
| Silver fractions {arginine | — | 2·1 |
| Histidine fraction after removal of carnosine nitrate | 37·5 | |
| After drying with baryta and extracting with alcohol {1st extract | 12 | |
| After drying with baryta and extracting with alcohol {2nd extract | — | 2·4 |
| After precipitation with picric acid | 10 | |
| Not precipitated by picric acid | — | 2 |
| Histamine isolated as pure picrate | 3 | |

As already mentioned, the complexity of muscle extracts is such that it could not be expected that the yields of histamine in the various stages would compare favourably with those obtained from extracts of lung or liver. They were, in fact, poor; but when the various stages are examined separately it will be seen that the yields are in most cases as high as can be expected under the circumstances.

The greatest discrepancy occurs during the phosphotungstic acid stage when only a 41 % yield was obtained. 3·75 mg. were left in the acetone-insoluble fraction, 8 mg. were found in the barium phosphotungstate precipitate and 7 mg. in the liquor from which the phosphotungstates had been precipitated. The other residues, except the potassium sulphate, were inactive. That is, a total of 18·75 mg. or 13 % can reasonably be ascribed to loss due

to adsorption on the bulky precipitates or solubility in the large volumes. This leaves 46 % unaccounted for. During this stage 76 g. potassium sulphate were removed. This, of course, could not be tested for histamine physiologically, and it has to be decided whether the values obtained in the first two stages, *i.e.* 140 mg., are (*a*) the true values, in which case, presumably, 46 % of the histamine was carried down with the potassium sulphate, or (*b*) values enhanced by the presence of large quantities of potassium salts exerting a depressant action on the cat's blood-pressure. In the latter case the yield obtained from the phosphotungstates would be more reasonable. A combination of these circumstances would provide a justifiable and probable explanation of the relatively large loss at this stage. In any case there is no definite evidence that fresh muscle tissue contains any organic depressor substance other than histamine. It may be noted in this connection that neither chemical nor physiological examination revealed the presence of choline in the muscle extracts examined.

The yields of depressor material during the silver fractionation and separation of carnosine nitrate are sufficiently high to require no comment when the large amounts of accompanying material are taken into consideration.

The yield during the alcohol extraction was 33 % in one extraction. This too is reasonable when it is remembered that the baryta mass still contained relatively large amounts of substances of an amino-acid nature which, as was shown by the preliminary experiments, definitely hinder the extraction.

Of the 12 mg. obtained in the alcoholic extract, which contained approximately 7 g. of material, 10 mg. were precipitated as picrate. This can, under the circumstances, be regarded as a good yield. In the long fractionation involved in the separation of pure histamine picrate a greater yield than 33 % could hardly be expected.

When viewed in this light the 4 % yield of pure histamine actually obtained is much better than the actual figure would suggest, for this yield is the result of a number of small losses in a very large number of operations and not due to an unreasonably large loss at any particular stage.

Although it cannot be claimed that it has been decisively proved, it seems justifiable to conclude that the whole of the depressor activity of an alcoholic extract of muscle prepared and tested under these conditions is due to the well-known base histamine.

Finally, I wish to express my gratitude to Dr H. W. Dudley for his valuable advice and criticism and his help during the large scale operations, to Dr H. H. Dale for his constant encouragement and interest, and to the latter, Dr J. H. Gaddum, Prof. O. S. Gibbs and Prof. A. N. Richards, for their kindness in performing the numerous blood-pressure tests required during this work.

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# XVI. A RAT TECHNIQUE FOR DEMONSTRATING THE INTERFERING EFFECT OF CEREALS ON BONE CALCIFICATION.

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E. Mellanby [1922] found that cereals interfere actively with the calcification of bones of dogs and that this action could be completely antagonised by a sufficiency of antirachitic vitamin (vitamin D) in the diet. The intensity of the anticalcifying influence varied greatly with the type of cereal, being greatest in the case of oatmeal and least in the case of white flour, other cereals such as maize, barley, rye, whole meal flour and rice occupying an intermediate position. The experimental results obtained were of greater interest because they were contrary to all previous supposition. The basis for the generally accepted views on the subject was undoubtedly that since oatmeal contained more calcium and phosphorus than, for instance, white flour, it would be expected to be more beneficial to the laying down of these elements in bone. The experiments on dogs completely reversed this expectation. The subject has been continuously followed up by one of us, using puppies as experimental animals, and has formed the basis of publications in which attempts at explaining the phenomena and conditions influencing the action have been described [Mellanby, E., 1924, 1925, 1926]. This work has proved very tedious and slow for various reasons. In the first place, the constituents of cereals are difficult to separate chemically or physically, especially on the scale necessary for the feeding of dogs. The second difficulty is that many of the simple forms of treatment of the cereals destroy the specific toxic effect, and thirdly, only a limited number of experiments, all of a prolonged nature, can be carried out with success at one time.

It was therefore thought advisable to develop a technique which would allow the study of this subject to be pursued on rats. If successful it would obviously simplify matters greatly, and by allowing the use of smaller quantities of prepared material, larger numbers of experiments could be carried out and results obtained more rapidly. It may be remembered that, although experiments on dogs demonstrating the presence of the antirachitic vitamin (now called vitamin D) were first described in 1918 [Mellanby, E., 1918], the general acceptance of this substance as an entity was delayed until other workers obtained confirmatory evidence of its presence by rat experiments in 1921 [McCollum, Simmonds, Shipley and Park, 1921; Korenchevsky, 1921].

It seemed likely that the demonstration of the toxic effects of cereals on rats would not only hasten the acceptance of the facts, but would also attract a larger number of workers to an aspect of nutrition which is probably as important as that of the vitamins, and would thus result in a more rapid identification and isolation of the factor or factors.

The object, therefore, of the present publication is primarily to show that the experimental results on dogs previously published can, in general, be demonstrated with equal ease in rats. No attempt has been made to go beyond this demonstration.

While the present work was being carried out, Dr P. Holst informed us privately that he had been able to demonstrate the interfering effect of cereals on calcification in rats by feeding them entirely on the individual cereals to be tested. It is obvious that such an experimental method must be of limited value, for animals under such conditions lose weight and this must interfere essentially with what is in reality an abnormality of the growth process. In Holst's [1927] results which have now been published the loss of weight of the animals is a prominent feature. Although his results can be regarded as confirming the anticalcifying action of cereals, the loss of weight of the animals prevents them from having a quantitative value. In the technique described below it will be seen that the animals grow rapidly, eat the food well and generally remain in a healthy condition over the required experimental period. Great differences in bone calcification are produced by different cereals, and by the same cereal after different forms of treatment.

*Feeding technique.*

The results obtained by experiments on dogs made it clear that in order to demonstrate the interfering effect of cereals on the calcification of bones at least two conditions were necessary, (1) that the diet should be deficient in vitamin D and (2) that the diet should not have a high calcium content. In the dog experiments it was shown that a sufficiency of vitamin D completely antagonised even the most powerful cereal effect and allowed the production of perfect bones. The addition of calcium carbonate to diets deficient in antirachitic vitamin improved calcification and tended to convert a rachitic to an osteoporotic condition of the bones. With a diet only slightly deficient in vitamin D, the addition of calcium carbonate often resulted in well calcified bones [Mellanby, E., 1925]. It seemed probable that additional calcium in the diet would be even more potent in antagonising the cereal effect in rats than in dogs, especially if rachitic pathological changes were used as criteria of abnormal calcification. It will be remembered that the development of standard rickets-producing diets in rats by McCollum, Simmonds, Shipley and Park [1921], Korenchevsky [1921] and Sherman and Pappenheimer [1921] necessitated an abnormal calcium-phosphorus ratio as well as a deficiency of the antirachitic vitamin in the diet. In the experimental diets chosen, although the calcium intake was relatively low as

compared with the phosphorus, it must be emphasised that there was no absolute deficiency, for the addition of vitamin D to the diet resulted in the development of well calcified bones without the addition of any more calcium.

In making up the synthetic diet which obviously had to contain a large proportion of cereal or cereal product, the following points were also taken into consideration. (1) The protein intake should be increased above that supplied by the cereal. This was done by adding caseinogen. (2) Substances in which cereals are deficient, namely sodium chloride, vitamin A and vitamin C, should be added. As a source of vitamin A dried cabbage was used, and lemon juice for vitamin C. (3) Since some of the cereal products tested were deficient in vitamins $B_1$ and $B_2$, an excess of these should be assured by the addition of marmite.

The basal diets were made up in the following way. A standard mixture was made containing caseinogen, 24 %; sodium chloride, 12 %; marmite, 32 %; lemon juice, 32 %. To 200 g. of this mixture were added 50 g. of dried cabbage. In the earlier experiments only 20 and 25 g. of dried cabbage were added, but in order to ensure good growth and complete freedom from ophthalmia this was ultimately increased to 50 g. 2·5 g. of this mixture was added to 7·5 g. of the cereal or cereal product to be tested and given to each young rat daily. During the early days of the experimental feeding less than this quantity was eaten. The caseinogen was heated at 120° for 36 hours in an electric oven before being added to the diet.

The diet contained only a small amount of vitamin D, dried cabbage being the main source of this, and the amount present was too little to prevent subnormal calcification whatever cereal was added. The same batch of dried cabbage was used in each series of experiments, so that the amount of vitamin D was constant where the results had to be compared. As regards calcium and phosphorus present in the diet, these varied with the type of cereal, both being higher when oatmeal or cereal germ was eaten than when white flour or rice formed the cereal basis. However, the increase in phosphorus was greater than that of calcium, since both in oatmeal and cereal germ the Ca : P ratio was less than that of white flour.

(1) *Duration of feeding experiment.* Young rats of weight between 30 and 40 g. were used. In each series of experiments the duration of feeding was the same but the period varied in different series between 17 and 52 days. The duration of any experiment was decided by a number of factors: (*a*) the importance of getting good differentiation between the calcification in the members of a series; under favourable conditions the longer the experimental period the better is the differentiation produced; (*b*) when, however, a powerful rickets-producing diet is eaten, as for instance when oatmeal forms the cereal basis and there is little or no vitamin D or extra calcium, then, although good growth is obtained in the first part of the experiment, this is often followed at a later period by loss of appetite and weight. Rats respond rapidly to this loss of weight and as in dogs [Mellanby, E., 1918] so in rats the rickety changes

in the bones are decreased and the calcium content increased, thus reducing the value of the experimental results. The experiment should, therefore, be ended, if possible, during the period of growth and not prolonged if the animals begin to lose weight.

(2) *Rate of growth.* Just as it is important to prevent loss of weight of the animal during the experimental period, so is it desirable to maintain, if possible, a fairly equal rate of growth in all the animals in a series. This is difficult, for it is found that after the first few weeks the appetite of the animals eating the diets with white flour as the cereal is much greater than that of the animals whose cereal basis is oatmeal. The amount of food eaten should therefore be regulated with the object of getting equal growth throughout. The increase in weight of the animals in some of the experiments described below was not as even as was desirable. It is hoped that this object will be more nearly obtained in future by closer control of the total intake of food by each rat.

(3) *Calcium content of bones.* E. Mellanby [1921] pointed out that the variations of the fat content of the bone marrow militated against the reliability of interpreting the calcium content of bones in terms of their dried weight. On the other hand, the great variations in size of bones of different animals necessitated due regard to bone weight. In dogs the ratio of calcium content to the original wet weight of a bone is a more reliable index of calcification than the ratio of calcium content to dried bone weight. The error due to fat content is particularly important in cases where the animals are either losing weight or gaining but slowly at the end of the experiment, for under these conditions fat disappears rapidly from the bone marrow. This difficulty has been overcome, in so far as the interpretation of calcification of rats' bones is concerned, by Chick, Korenchevsky and Roscoe [1926], who extracted the fat of the bone before drying and weighing. This method has been adopted in the present experiments. The calcium percentage is given in terms of the weight of the dried fat-extracted bones. The $A/R$ ratio described by Chick, Korenchevsky and Roscoe is also given in the tables below. $A$ represents the ash of the bones and $R$ the difference between the weights of the fat-extracted dried bone and the ash. The bones used for calcium estimation were the femur, tibia and fibula of one leg.

## Experimental Results.

### I. *The effect of increasing the cereal relatively to the other substances in the diet.*

As in the case of dogs [Mellanby, E., 1920, 1921, 1925], increasing the proportion of cereal eaten and keeping other substances in the diet constant brings about worse calcification of the bones. The following experimental results (Table I) illustrate this fact. In the tables basal diet means the basal substances described above without the added cereal.

Table I.

| No. of exp. | Duration in days | Cereal added to basal diet | Sex | Init. weight g. | Max. weight g. | % increase weight | Ca in bones: % of dry weight after fat extraction | Ca in bones: $A/R$ ratio |
|---|---|---|---|---|---|---|---|---|
| 79 | 31 | 3·75 g. white flour | F. | 44 | 47 | 7 | 18·07 | 0·95 |
| 80 | 31 | ,, | M. | 50 | 67 | 34 | 19·34 | 1·09 |
| 82 | 31 | 7·5 g. white flour | M. | 47 | 74 | 57 | 17·61 | 0·85 |
| 83 | 31 | ,, | M. | 33 | 70 | 112 | 15·48 | 0·72 |
| 84 | 31 | ,, | M. | 49 | 94 | 92 | 16·39 | 0·82 |

It will be seen that doubling the amount of white flour in 82, 83 and 84 reduced the average $A/R$ ratio from 1·02 (79 and 80) to 0·80 (82, 83 and 84). Of course, the increase in cereal brought about increased rate of growth, but that this in itself was not entirely responsible for the more defective calcification is evident from the comparative effect of different cereals described below. As has already been pointed out in puppies [Mellanby, E., 1922, 1925], although equal growth may be produced by different cereals, there is great variation in calcification.

### II. *The effect of different cereals on calcification.*

The method described above of feeding rats also allows great differences in the anticalcifying effect of different cereals to be demonstrated. The results are, on the whole, in agreement with those previously obtained by experiments on dogs. Some examples of experimental results are given in Table II.

Table II.

*Series* 1.

| No. of exp. | Duration in days | Cereal added to basal diet | Sex | Init. weight g. | Max. weight g. | % increase weight | Ca in bones: % of dry weight after fat extraction | Ca in bones: $A/R$ ratio |
|---|---|---|---|---|---|---|---|---|
| 35 | 28 | White flour | M. | 37 | 106 | 186 | 17·41 | 0·85 |
| 37 | 28 | Oatmeal | M. | 33 | 66 | 100 | 14·08 | 0·61 |
| 39 | 28 | Wholemeal flour | M. | 40 | 95 | 138 | 15·83 | 0·75 |
| 45 | 28 | Maize meal | M. | 34 | 77 | 126 | 17·10 | 0·86 |
| 47 | 28 | Barley meal | M. | 31 | 90 | 180 | 18·31 | 0·98 |
| 49 | 28 | Rice | M. | 31 | 73 | 135 | 16·67 | 0·82 |

*Series* 2.

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 36 | 42 | White flour | F. | 30 | 95 | 217 | 18·85 | 1·04 |
| 38 | 42 | Oatmeal | F. | 35 | 84 | 140 | 15·34 | 0·70 |
| 40 | 42 | Wholemeal flour | F. | 31 | 82 | 164 | 14·94 | 0·67 |
| 46 | 42 | Maize meal | F. | 31 | 72 | 132 | 16·24 | 0·78 |
| 48 | 42 | Barley meal | F. | 29 | 96 | 265 | 19·60 | 1·13 |
| 50 | 42 | Rice | F. | 32 | 68 | 112 | 15·22 | 0·70 |

It will be noticed that the first series lasted 28 days and the second series 42 days.

There is fair agreement in the results of the two series. Oatmeal and wholemeal flour have brought about the worst calcification of the bones, while barley meal and white flour are associated with the best calcified bones.

The first of the following series (Table III) also emphasises the relatively potent anticalcifying action of oatmeal as compared with white flour.

Table III.

| No. of exp. | Duration in days | Cereal added to basal diet | Sex | Init. weight g. | Max. weight g. | % increase weight | Ca in bones: % of dry weight after fat extraction | Ca in bones: $A/R$ ratio |
|---|---|---|---|---|---|---|---|---|
| 56 | 42 | White flour | F. | 35 | 81 | 131 | 15·60 | 0·73 |
| 57 | 42 | ,, | F. | 33 | 81 | 145 | 15·93 | 0·75 |
| 58 | 42 | Oatmeal | F. | 34 | 77 | 126 | 13·11 | 0·63 |
| 59 | 42 | ,, | M. | 33 | 68 | 106 | 13·04 | 0·59 |
| 117 | 30 | White flour | M. | 37 | 65 | 76 | 16·94 | 0·81 |
| 120 | 30 | Rice | M. | 52 | 57 | 10 | 16·23 | 0·78 |
| 122 | 30 | ,, | M. | 38 | 51 | 34 | 16·11 | 0·77 |

The last two experiments (120 and 122) in which rice was the cereal eaten are given to illustrate one of the difficulties met with in these experiments. It is the most obvious instance in which this technique at present fails because in the majority of cases in which rice is used the growth obtained is poor and so does not allow a good comparison with other cereals to be made. Why the growth should be so relatively poor in the rice experiments is not known. If starch be used instead of a cereal, very poor growth also results, as is seen in the next experiments (Table IV).

Table IV.

| No. of exp. | Duration in days | Cereal added to basal diet | Sex | Init. weight g. | Max. weight g. | % increase weight | Ca in bones: % of dry weight after fat extraction | Ca in bones: $A/R$ ratio |
|---|---|---|---|---|---|---|---|---|
| 1 | 52 | White flour | M. | 49 | 113 | 131 | 17·24 | 0·89 |
| 6 | 52 | Starch | F. | 39 | 46 | 18 | 17·02 | 0·91 |
| 7 | 52 | ,, | F. | 30 | 50 | 67 | 17·06 | 0·94 |
| 12 | 52 | Oatmeal | F. | 29 | 60 | 107 | 13·06 | 0·60 |

The low calcium content of the bones of Exps. 56 and 57 of Table III, where the animals ate white flour as the cereal basis, requires comment. The probable reason for these results as compared with those obtained in animals eating similar diets and recorded in other tables is that the vitamin D content of the cabbage eaten was smaller than usual. Since all the animals of the series received the same cabbage, the individual results are comparable with each other although not with those of other series.

The results obtained with starch not only show that, under these dietetic conditions, the growth of the animals is poor, but suggest that the carbohydrate moiety of the cereal is not responsible for the anticalcifying effect. This is confirmatory of the results on dogs [Mellanby, E., 1922, 1925].

In the dog experiments previously referred to [Mellanby, E., 1925] it was found that wheat germ also interfered with bone calcification. This fact can also be seen in rat experiments (Table V).

Table V.

| No. of exp. | Duration in days | Cereal added to basal diet | Sex | Init. weight g. | Max. weight g. | % increase weight | Ca in bones: % of dry weight after fat extraction | Ca in bones: *A/R* ratio |
|---|---|---|---|---|---|---|---|---|
| 119 | 24 | White flour | M. | 39 | 75 | 90 | 17·37 | 0·89 |
| 124 | 24 | 73 % white flour and 27 % wheat germ | M. | 32 | 68 | 119 | 14·94 | 0·67 |
| 127 | 24 | 73 % white flour and 27 % maize germ | F. | 32 | 52 | 62 | 15·95 | 0·76 |
| 118 | 30 | White flour | M. | 57 | 100 | 75 | 17·76 | 0·92 |
| 123 | 30 | 73 % white flour and 27 % wheat germ | M. | 38 | 74 | 94 | 12·76 | 0·52 |
| 126 | 30 | 73 % white flour and 27 % maize germ | F. | 28 | 53 | 89 | 15·32 | 0·71 |
| 128 | 30 | 73 % white flour and 27 % maize germ | M. | 34 | 47 | 38 | 15·41 | 0·71 |

In these experiments 27 % of either wheat or maize germs was substituted for 27 % of the white flour. It is evident that both types of germ interfere with bone calcification.

### III. *Influence of salts containing calcium and phosphorus on the cereal effect.*

In the dog experiments it was found that the effect of the cereals could be modified by altering the calcium and phosphorus of the diet [Mellanby, E., 1925]. The addition, for instance, of calcium carbonate, especially to a diet containing oatmeal as cereal, tended to improve calcification and convert rachitic changes of bones into osteoporosis. The improvement in calcification was much less than that produced by adding sources of antirachitic vitamin. Calcium phosphate, in the absence of vitamin D, produced a smaller improvement than calcium carbonate. The importance of these observations was that they affected the question as to the cause of the cereal effect. One of the most obvious suggestions as to causation was that the anticalcifying influence of cereals was due to their calcium-phosphorus ratio, for both oatmeal and wheat germ have a relatively low calcium-phosphorus ratio as compared with white flour. Since, also, raising the ratio by adding calcium carbonate decreased the rickets-producing effect, the hypothesis that the action of cereals could be explained on the basis of this ratio was strengthened. The problem of the part played by the calcium-phosphorus balance in the aetiology of rickets and bone calcification has loomed large in experimental work since its importance in experiments on rats was pointed out by McCollum, Simmonds, Shipley and Park [1921]. The whole question from the point of view of cereals was considered by one of us and the conclusion was reached that the balance of evidence did not yet justify the belief that this simple explanation covered all the facts [Mellanby, E., 1925]. This point, however, is still not settled. The following experiments with rats (Table VI) show that the addition of calcium salts antagonises the anticalcifying effect of cereals in the absence or great deficiency of vitamin D. They differ from the results obtained

with dogs in that calcium phosphate seems to bring about as great an improvement in calcification as calcium carbonate. It may be remembered that in the dog experiments this only happened when a source of fat-soluble vitamin such as butter was included in the diet.

Table VI.

| No. of exp. | Duration in days | Cereal and salt added to basal diet | Sex | Init. weight g. | Max. weight g. | % increase weight | Ca in bones: % of dry weight after fat extraction | Ca in bones: *A*/*R* ratio |
|---|---|---|---|---|---|---|---|---|
| | | *Series* 1. | | | | | | |
| 94 | 17 | Oatmeal | F. | 35 | 77 | 120 | 16·49 | 0·78 |
| 96 | 17 | ,, | M. | 40 | 85 | 111 | 16·11 | 0·77 |
| 102 | 17 | Oatmeal + 0·5 % $CaCO_3$ | M. | 40 | 81 | 103 | 18·05 | 0·95 |
| 103 | 17 | ,, | F. | 52 | 106 | 104 | 18·91 | 1·04 |
| 107 | 17 | Oatmeal + 0·5 % $Ca_3(PO_4)_2$ | F. | 38 | 76 | 100 | 17·81 | 0·92 |
| 108 | 17 | ,, | M. | 44 | 86 | 95 | 17·20 | 0·87 |
| 109 | 17 | Oatmeal + 0·5 % $K_3PO_4$ | M. | 42 | 73 | 74 | 15·57 | 0·72 |
| 112 | 17 | ,, | F. | 55 | 90 | 64 | 17·10 | 0·86 |
| | | *Series* 2. | | | | | | |
| 93 | 24 | Oatmeal | F. | 30 | 66 | 120 | 16·48 | 0·80 |
| 101 | 24 | Oatmeal + 0·5 % $CaCO_3$ | M. | 35 | 78 | 123 | 17·77 | 0·92 |
| 105 | 24 | Oatmeal + 0·5 % $Ca_3(PO_4)_2$ | M. | 32 | 68 | 113 | 17·81 | 0·93 |
| 110 | 24 | Oatmeal + 0·5 % $K_3PO_4$ | M. | 25 | 57 | 128 | 17·24 | 0·87 |
| | | *Series* 3. | | | | | | |
| 95 | 30 | Oatmeal | M. | 42 | 94 | 124 | 15·41 | 0·71 |
| 104 | 30 | Oatmeal + 0·5 % $CaCO_3$ | M. | 38 | 93 | 145 | 18·03 | 0·95 |
| 106 | 30 | Oatmeal + 0·5 % $Ca_3(PO_4)_2$ | F. | 48 | 100 | 108 | 19·50 | 1·11 |
| 111 | 30 | Oatmeal + 0·5 % $K_3PO_4$ | F. | 35 | 63 | 80 | 15·63 | 0·73 |

These experimental results show that the addition of either calcium carbonate or calcium phosphate to the diet improves calcification and that there is little or no difference between the action of the two salts. That it is the calcium which is important is also evident from the fact that potassium phosphate had much less effect on the calcification of the bone. The effect of potassium phosphate in these experiments was somewhat variable; in two of the above cases there was slight improvement, in one slight impairment of calcification and in one case no effect. At all times the effect was small. It would appear that the addition of calcium is of more importance in itself than because of any influence the added salts have on the calcium-phosphorus ratio. If this ratio were crucial it would be expected that the addition of potassium phosphate would have made bone calcification consistently worse, but this is not the case.

The figures in Table VII represent the approximate calcium and phosphorus amounts and the calcium-phosphorus ratios of the above diets.

Table VII.

| Addition to basal diet | Ca % | P % | Ca/P ratio | Effect on bone calcification |
|---|---|---|---|---|
| 1. Oatmeal | 0·205 | 0·47 | 0·42 | Very bad |
| 2. Oatmeal and $CaCO_3$ | 0·35 | 0·47 | 0·74 | Much better than 1 |
| 3. Oatmeal and $Ca_3(PO_4)_2$ | 0·32 | 0·58 | 0·55 | Like 2 and much better than 1 |
| 4. Oatmeal and $K_3PO_4$ | 0·20 | 0·58 | 0·34 | Similar to 1 |

### IV. *The antagonism of the cereal effect by vitamin D.*

Even the most potent anticalcifying effect of cereal, such as that produced by oatmeal, can be overcome by a sufficiency of the antirachitic vitamin in dogs [Mellanby, E., 1925]. The following experiments (Table VIII) show the effect of adding 2·5 and 5 mg. of cod-liver oil daily to the basal diets containing oatmeal.

Table VIII.

| No. of exp. | Duration in days | Cereal and oil added to basal diet | Sex | Init. weight g. | Max. weight g. | % increase weight | Ca in bones: % of dry weight after fat extraction | Ca in bones: *A/B* ratio |
|---|---|---|---|---|---|---|---|---|
| | | | *Series* 1. | | | | | |
| 88 | 25 | Oatmeal | F. | 50 | 72 | 44 | 16·25 | 0·78 |
| 89 | 25 | Oatmeal + 2·5 mg. cod-liver oil | M. | 45 | 83 | 84 | 17·80 | 0·96 |
| | | | *Series* 2. | | | | | |
| 94 | 17 | Oatmeal | F. | 35 | 77 | 120 | 16·49 | 0·78 |
| 96 | 17 | „ | M. | 40 | 85 | 111 | 16·11 | 0·77 |
| 98 | 17 | Oatmeal + 5 mg. cod-liver oil | M. | 34 | 69 | 103 | 18·08 | 0·96 |
| 99 | 17 | „ | M. | 35 | 73 | 109 | 18·15 | 0·96 |
| | | | *Series* 3. | | | | | |
| 93 | 24 | Oatmeal | F. | 30 | 66 | 120 | 16·48 | 0·80 |
| 100 | 24 | Oatmeal + 5 mg. cod-liver oil | F. | 38 | 99 | 161 | 19·71 | 1·14 |
| | | | *Series* 4. | | | | | |
| 95 | 30 | Oatmeal | M. | 42 | 94 | 124 | 15·41 | 0·71 |
| 97 | 30 | Oatmeal + 5 mg. cod-liver oil | M. | 36 | 119 | 231 | 19·38 | 1·10 |

These figures show that even 2·5 mg. of cod-liver oil bring about some improvement in calcification, while 5 mg. result in great improvement.

### V. *The irradiation of oatmeal by means of a mercury vapour lamp.*

The diminution of the rickets-producing effect by irradiating cereals as shown in dogs [Mellanby, E., 1925], can also be demonstrated in rats by the above technique. In the experiments in Table IX fine oatmeal was exposed to the

rays of a mercury vapour lamp for 30 minutes at a distance of a foot. The oatmeal was scattered on plates and stirred frequently during the time of exposure.

Table IX.

| No. of exp. | Duration in days | Cereal added to basal diet | Sex | Init. weight g. | Max. weight g. | % increase weight | Ca in bones: % of dry weight after fat extraction | Ca in bones: $A/R$ ratio |
|---|---|---|---|---|---|---|---|---|
| 66 | 28 | Oatmeal | M. | 44 | 97 | 143 | 15·78 | 0·66 |
| 77 | 28 | Irradiated oatmeal | M. | 40 | 102 | 155 | 17·38 | 0·88 |
| 64 | 35 | Oatmeal | M. | 42 | 80 | 90 | 13·45 | 0·57 |
| 65 | 35 | „ | M. | 31 | 72 | 132 | 14·45 | 0·63 |
| 76 | 35 | Irradiated oatmeal | M. | 32 | 97 | 203 | 17·58 | 0·90 |
| 78 | 35 | „ | M. | 30 | 95 | 216 | 18·93 | 1·05 |

In view of recent work, especially that of Rosenheim and Webster [1927] and of Hess and Windaus [1927], there can be little doubt that the improvement in calcification of bones brought about by irradiating oatmeal is due to the formation of vitamin D (antirachitic vitamin) from its parent non-active substance, ergosterol, in the oatmeal.

VI. *The destruction of the anticalcifying action of cereals by acids.*

It has been shown above that vitamin D (antirachitic vitamin), whether given in foods containing it or produced in the food by exposure to a source of ultra-violet radiation, antagonises the anticalcifying action of cereals. In the experiments now to be described the rickets-producing effect of cereals was reduced not by the addition of an antagonising substance but by the actual destruction of the offending agent in the cereal by acid. The cereal was first boiled with 1 % hydrochloric acid until the mixture gave no colour with iodine, *i.e.* until the starch was hydrolysed. The mixture was then neutralised ($p_H$ 6) and added to the diets so as to correspond with the actual amount of cereal given to the control animals. In the control experiments, *i.e.* where oatmeal itself was eaten, an amount of NaCl was added equivalent to that contained in the diets in which the oatmeal had been boiled with hydrochloric acid and neutralised (Table X).

Table X.

| No. of exp. | Duration in days | Cereal added to basal diet | Sex | Init. weight g. | Max. weight g. | % increase weight | Ca in bones: % of dry weight after fat extraction | Ca in bones: $A/R$ ratio |
|---|---|---|---|---|---|---|---|---|
| 58 | 42 | Oatmeal | F. | 34 | 77 | 126 | 13·11 | 0·63 |
| 59 | 42 | „ | M. | 33 | 68 | 106 | 13·04 | 0·59 |
| 60 | 42 | „ | F. | 37 | 53 | 72 | 15·02 | 0·69 |
| 61 | 42 | Acid oatmeal | M. | 35 | 68 | 94 | 17·86 | 0·93 |
| 62 | 42 | „ | F. | 35 | 75 | 114 | 17·87 | 0·93 |
| 63 | 42 | „ | M. | 36 | 80 | 122 | 18·32 | 1·00 |

It is clear from these results that boiling oatmeal with 1 % acid in this way destroys to a great extent its power to interfere with bone calcification. It is important to note that the time of boiling and the strength of the acid

used are of great significance. Holst [1927] has indeed found that the rickets-producing effect of oatmeal can be obtained in a filtrate obtained by boiling this cereal with 0·5 % hydrochloric acid. Thus, whereas a short period of boiling with dilute acid brings the rickets-producing substances into solution, more prolonged boiling of the cereal destroys this substance. This part of the subject will be dealt with more fully in a subsequent paper.

## Conclusions.

The experimental work described above shows that it is possible by suitable dietetic methods to demonstrate in rats the anticalcifying actions of cereals similar to those previously demonstrated by one of us in dogs [Mellanby, E., 1922, 1925, 1926]. The feeding technique, although undoubtedly capable of improvement, allows good growth and, except in the worst rickets-producing diets, fair health during the experimental period. It has been found possible (*a*) to make the intensity of rickets worse by increasing the cereal intake when the other dietetic ingredients are kept constant; (*b*) to demonstrate great differences in the rickets-producing effects of different cereals, oatmeal having the most potent and white flour the least action in this respect; (*c*) to demonstrate the rickets-producing effect of germ, both of wheat and maize; (*d*) to show the antagonism of substances containing vitamin D to the cereal action or to lessen the effect by exposing the cereal to ultra-violet radiations; (*e*) to show that raising the calcium of the diet either by adding calcium carbonate or calcium phosphate minimises the anti-calcifying action of cereals; and (*f*) to demonstrate the ultimate destruction of the anticalcifying action of cereals by boiling with dilute hydrochloric acid.

What constituent of the cereal is responsible for this powerful property of interfering with bone calcification has not been discussed in this paper, but the simplified technique described ought to allow a more rapid accumulation of facts concerning this important side of dietetics.

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# XVII. STUDIES ON XANTHINE OXIDASE. X.
## THE ACTION OF LIGHT.

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The action of light on various enzymes has been studied by many investigators. In the great majority of cases the light has been found to produce a destruction of the enzyme, particularly in the presence of certain dyes. The enzymes which have been chiefly studied are invertase, diastase, pepsin, trypsin, zymase, urease, peroxidase, and catalase. In all these cases destruction has been found to occur. The presence of oxygen appears however to be necessary for the action of light. Tappeiner and Jodlbauer [1905] found that invertase and diastase underwent no destruction in the absence of oxygen, and Jamada and Jodlbauer [1908] and Zeller and Jodlbauer [1908] showed that this was also true of peroxidase and catalase respectively. These authors have found, however, that although oxygen is necessary in all these cases for the action of visible light, the destruction by ultra-violet light is independent of the presence of oxygen.

The action of light on the reducing enzymes of the dehydrase type has not been systematically investigated. In fact the only reference in the literature appears to be a statement in a paper by Harden and Norris [1915] on the reduction of methylene blue by the enzymes of dried yeast. They stated that the reduction was greatly accelerated in certain cases by exposure to light, but that this acceleration only occurred if the solutions contained dissolved oxygen.

We have investigated the effect of visible light on the reduction of methylene blue by the xanthine oxidase system. As the source of the enzyme we used either fresh milk, the caseinogen preparation of Dixon and Thurlow [1924], or the whey preparation of Dixon and Kodama [1926]. The reaction was carried out in all cases in glass Thunberg tubes in the usual manner.

### The action of light.

On comparing the rates of reduction of methylene blue by the xanthine oxidase system in the dark and in sunlight, effects were observed precisely similar to those obtained by Harden and Norris with the yeast enzymes. If the tubes were carefully freed from oxygen by thorough evacuation, shaking to remove all dissolved gases, refilling with nitrogen, and once more thoroughly evacuating, no acceleration was produced by the sunlight. If, however, the tubes were simply exhausted to a pressure of 5 cm. of Hg, so as to leave a

small amount of oxygen, the light produced a large acceleration. This is illustrated by the following experiment.

*Exp.* 1. Each tube contained 3 cc. of a 2 % solution of the whey preparation + 2 cc. phosphate buffer ($p_H$ 7·6) + 1 cc. 1/5000 methylene blue + 0·1 cc. of a 0·2 % solution of hypoxanthine.

| | Reduction times at 15° | |
|---|---|---|
| | In sunlight | In dark |
| Oxygen-free | 10′ | 10′ |
| Air at 5 cm. Hg. | 15′ | 45′ |

The apparent rate of reduction is naturally slower in the presence of the air owing to the reoxidation of the leuco-methylene blue by the oxygen, but it will be seen that in the presence of the air the reaction is accelerated threefold by the light. The experiment shows clearly that the presence of oxygen is necessary for the action of light.

Various sources of illumination were tried; sunlight, a mercury arc lamp, a carbon arc, and even an ordinary 100 watt electric bulb at a distance of 1 ft.; all gave large accelerations.

A very slight rise of temperature during the irradiation could not always be avoided, but it was always too insignificant to affect the reduction time appreciably, and the irradiation was never found to produce any effect in the absence of oxygen. In experiments where care was taken to keep the temperature absolutely constant during the irradiation similar results were obtained.

The next stage in the investigation of the action of light on the reaction was to determine the effect of previous irradiation of the components of the system individually in the presence of oxygen, the actual reaction being subsequently carried out in the absence of light and oxygen.

It was found that previous irradiation of the buffer solution or the hypoxanthine produced no effect on the reaction velocity. A brief preliminary irradiation of the enzyme (in the presence of oxygen) alone or, better, in the presence of methylene blue greatly increases its activity as the following experiment shows.

*Exp.* 2. The same quantities were used as in Exp. 1 but with a different preparation of the oxidase. In tube A none of the constituents was irradiated. In tube B the enzyme and buffer were previously exposed to sunlight in a tube open to the air for half an hour, and the methylene blue and hypoxanthine subsequently added. In tube C the methylene blue was added to the enzyme and buffer and then exposed similarly to sunlight for half an hour, the hypoxanthine being added afterwards. In this and in all subsequent experiments all the tubes were cooled to the same temperature after irradiation, and immediately after adding the hypoxanthine all traces of oxygen were very carefully removed from the tubes by the methods used in Exp. 1. Errors due to temperature differences and reoxidation of the leuco-methylene blue were therefore eliminated.

| Tube | Reduction time in dark at 15° |
|---|---|
| A | 42′ |
| B | 35′ |
| C | 20′ |

It will be seen that the previous irradiation of the oxidase, particularly in the presence of the dye, has produced a large increase in its activity. The experiment is typical of many.

It was found that while short exposures produced an acceleration, longer exposures produced a deceleration, presumably owing to a destruction of the enzyme. This effect, as well as the acceleration, is dependent on the presence of oxygen, as the following experiment shows.

*Exp.* 3. The same quantities were used as in Exp. 1, except that only 0·5 cc. of methylene blue was taken, and that fresh milk was used instead of the solution of the oxidase preparation. (Precisely similar results have been obtained with the purified oxidase.) In tube A the components were not irradiated. In tubes B and C the milk, buffer and methylene blue were mixed and exposed to sunlight for 6 hours. Tube B was carefully freed from oxygen before the irradiation, while tube C was left open to the air and occasionally shaken during the irradiation. The day was cold and the temperature did not rise appreciably.

| Tube | Reduction time in dark at 37° |
|---|---|
| A | 3′ |
| B | 3·5′ |
| C | Not reduced in 60′ |

Eosin also was found to be capable of acting as a sensitising dye instead of methylene blue.

*Exp.* 3 *a*. The procedure of the preceding experiment was repeated exactly, except that 0·5 cc. 1/500 eosin was added before the irradiation instead of the methylene blue. The methylene blue was added after the irradiation and it was found that the determination of its reduction-time was a perfectly easy matter in spite of the presence of the eosin, which is not reduced by the system.

| Tube | Reduction time in dark at 37° |
|---|---|
| A | 3′ |
| B | 5′ |
| C | Not reduced in 60′ |

It is clear that the nature of the effect (acceleration or deceleration) produced by the irradiation depends on the length of the period of exposure. The following experiment shows the relation more completely.

*Exp.* 4. A series of open tubes each containing 2 cc. of a 2 % whey preparation + 2 cc. buffer ($p_H$ 7·6) + 1 cc. methylene blue was irradiated by a carbon arc at a distance of 1 ft. At intervals tubes were removed and cooled (the temperature rose a few degrees during the irradiation), and the reduction times with hypoxanthine then determined at 15° after thorough evacuation. A second series with enzyme and buffer but without methylene blue was simultaneously irradiated, the methylene blue and hypoxanthine being added afterwards. The results are given graphically in Fig. 1. Fig. 2 gives a similar

curve obtained by exposing 0·5 cc. of fresh milk + 1 cc. methylene blue + 3 cc. buffer to strong sunlight for varying periods. In both cases the acceleration produced by short exposures and the destruction with long exposures is well marked.

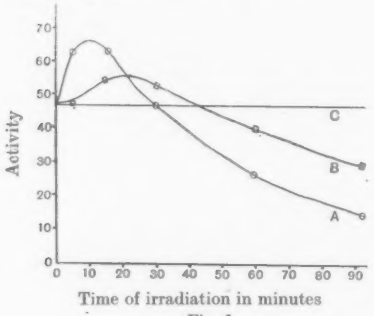


Fig. 1.

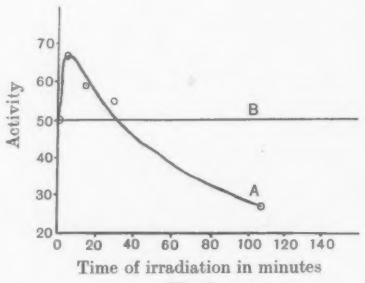


Fig. 2.

Fig. 1. Effect of previous irradiation of oxidase with carbon arc.
A. Enzyme irradiated with methylene blue in presence of air.
B. Enzyme irradiated without methylene blue in presence of air.
C. Enzyme irradiated with methylene blue in absence of air.
"Activity" denotes reciprocal of reduction-time (in minutes) × 100.

Fig. 2. Effect of previous irradiation of oxidase with sunlight.
A. Enzyme irradiated with methylene blue in presence of air.
B. Enzyme not irradiated.

Most of those who have studied the action of light on other enzymes have obtained only a destruction of the enzyme. In general it appears however that the exposures given were fairly long. In two cases the effect of short exposures has been studied, and in both cases results similar to those given above were obtained. Green [1897] found that exposing ptyalin to light at first markedly increased its activity, and then with longer exposures destroyed it. Bering and Meyer [1912] showed that exactly the same effects were produced in the case of peroxidase. It seems probable that the results obtained by Harden and Norris with the reducing enzymes of yeast may be due to a similar action. The fact that similar effects have been obtained with enzymes of such different origin and nature suggests that the phenomenon may be a fairly general one, and is a point of some interest in connection with the problem of enzyme constitution.

The fact that oxygen is necessary, as well as light, for both the phase of acceleration and the phase of destruction indicates that the two effects are due to the same cause—a photo-oxidation of the enzyme, the first stage of the oxidation resulting in an increase in its activity, possibly by the production of fresh active centres, the later stage causing its destruction.

It appeared possible from certain other considerations that the oxygen might form some active oxidising agent under the influence of the light in the presence of the dye, and that this agent might act on the enzyme to produce the effects observed.

The effect of previous irradiation of the methylene blue alone was therefore next tested in the presence and absence of oxygen.

*Exp.* 5. In each tube were placed 1 cc. methylene blue + 3 cc. buffer. Tube A was not irradiated. Tube B was left open to the air and was irradiated for 2 hours by the mercury vapour lamp. Tube C was thoroughly evacuated and then similarly irradiated. After the irradiation the tubes were cooled to the same temperature, 0·5 cc. milk (diluted 1 : 2) added, and the tubes allowed to stand for an hour. The reduction-times with 0·1 cc. hypoxanthine were then determined in the usual way.

| Tube | Reduction time in dark at 15° |
|---|---|
| A | 135′ |
| B | 165′ |
| C | 115′ |

It will be seen that the previous irradiation of the dye (in the presence of oxygen) has produced a distinct deceleration of the reaction so that the above hypothesis of the formation of the active agent from oxygen and the dye by the irradiation seems to be supported. If, however, the irradiated dye is not allowed to stand for some time with the enzyme before testing, no effect whatever is produced and the reduction-times with irradiated and non-irradiated dye are identical. The period of standing is necessary in order to allow the active oxidising agent to act upon the enzyme, as is shown in experiments given later.

The irradiation of the dye *in vacuo* resulted in a small acceleration (tube C). This acceleration is probably to be explained by the presence of a small trace of oxygen in the tube. This may have been sufficient for the formation of an amount of the oxidising substance too small to produce any destruction of the enzyme but enough to give a slight acceleration.

If the view expressed above is correct we should expect that a given amount of the active substance will have a destructive effect on a small amount of enzyme but an accelerating effect on larger amounts, since an amount sufficient to oxidise a small amount of enzyme as far as the phase of destruction will be insufficient to oxidise a larger amount beyond the acceleration phase. The following experiment, which is typical of several, shows that this is actually the case.

*Exp.* 6. Two tubes each containing 1 cc. methylene blue + 3 cc. buffer were irradiated 2½ hours with the mercury vapour lamp, being left open to the air during the irradiation. To one tube was then added 0·2 cc. milk and to the other 1 cc. milk. Two control tubes were also put up with similar quantities, but with non-irradiated dye. All four tubes were then allowed to stand for an hour at room temperature. 0·1 cc. hypoxanthine was then added, the tubes were carefully evacuated, and the reduction-times determined as before.

| | Reduction time in dark | | |
|---|---|---|---|
| | Dye irradiated | Dye not irradiated | |
| 0·2 cc. milk | 26′ | 23·5′ | (at 37°) |
| 1·0 cc. „ | 14′ | 19·5′ | (at 15°) |

With 0·2 cc. milk the previously irradiated dye has produced some destruction of the enzyme, but with five times as much milk it has produced a marked acceleration; thus on changing the enzyme concentration an actual reversal of the effect of the previous irradiation occurs.

The effect of varying the enzyme concentration is also evident in the following experiment in which the enzyme and the methylene blue were previously irradiated together.

*Exp.* 7. In this experiment open tubes containing 1 cc. methylene blue + 2 cc. buffer + 2 cc. of a solution of the whey preparation of the strength given in the first column of the table were irradiated for 16 minutes with a carbon arc, and the activities compared as before with similar non-irradiated control solutions.

| | Reduction times in dark at 15° | |
|---|---|---|
| | Previously irradiated | Not irradiated |
| 2 % whey preparation | 15′ | 16′ |
| 1 % ,, | 63′ | 35′ |
| 0·5 % ,, | No reduction in 2 hours | 45′ |

## The formation and action of $H_2O_2$.

Certain facts in connection with the behaviour of the irradiated dyes had led us to consider the possibility that the active agent, which was formed during the irradiation in the presence of oxygen and which then acted on the enzyme to produce the effects observed, might be hydrogen peroxide.

In order to test whether hydrogen peroxide is actually formed when a methylene blue solution is irradiated in the presence of oxygen we endeavoured to see whether nitrites could be oxidised by such a solution in the presence of milk peroxidase, using the technique of Thurlow [1925]. Hydrogen peroxide oxidises nitrite rapidly in the presence of such a peroxidase, but not at an appreciable rate in its absence.

*Exp.* 8. The peroxidase was prepared by the method given by Thurlow. The Griess-Ilosvay reagent was used to detect and estimate roughly the nitrite remaining at the end of the experiment. By comparing the resulting colour with the colour given under the same conditions by an amount of nitrite equal to that originally added it was possible to determine how much had been oxidised during the experiment. A careful series of preliminary experiments showed that this was perfectly feasible in spite of the presence of the methylene blue. The concentration of the latter was the same in all tubes, and the blue colour in no way interfered with the detection of the red colour produced by the Griess-Ilosvay reagent. Six tubes were taken. Tubes A, B, C and F contained 1 cc. of a 5 % solution of milk peroxidase + 2 cc. buffer + 0·5 cc. methylene blue + 1 cc. $NaNO_2$ (= 0·01 mg.). In tube D the peroxidase was previously inactivated by boiling, the contents being otherwise the same, while tube E contained peroxidase, buffer, and nitrite but no dye. Tube A was a control and was tested immediately after the mixing. It served

as a standard to indicate the colour given by the amount of nitrite originally present. Tubes C, D and E were irradiated open to the air for 1½ hours with the mercury vapour lamp. Tube F was similarly irradiated but with all traces of oxygen carefully removed from the tube. Tube B was not irradiated but stood meanwhile in the dark open to the air. At the end of the irradiation 0·5 cc. methylene blue was added to tube E and the Griess-Ilosvay reagent added to all tubes. The results were as follows:

| Tube | Nitrite remaining unoxidised |
|---|---|
| A | + + + + |
| B | + + + + |
| C | − − |
| D | + + + + |
| E | + + |
| F | + + + + |

In tube C which was irradiated and in which oxygen, dye and peroxidase were all present, the oxidation of the nitrite was complete. The presence of all factors is, however, necessary. No oxidation took place in the absence of light (tube B) or of oxygen (tube F) or with boiled peroxidase (tube D). Some oxidation indeed occurred in tube E where all the factors were present except the methylene blue. This is probably to be explained by the formation of a small amount of peroxide by the irradiated peroxidase preparation itself (compare the similar effect in Exp. 4).

We consider that these results constitute good evidence of peroxide formation during irradiation in the presence of oxygen.

It now remained to determine whether the effects of the irradiation on the xanthine oxidase could be duplicated by the action of hydrogen peroxide, as was to be expected if our view were correct that this was the agent responsible for the light effects.

It had already been shown [Dixon, 1925] that the xanthine oxidase, in common with most enzymes, was destroyed by small amounts of hydrogen peroxide. Still smaller amounts should, on our view, produce an increase in the activity of the enzyme. The action on the enzyme of hydrogen peroxide in varying amounts was therefore tested.

*Exp.* 9. A series of tubes was prepared containing 5 cc. buffer + 1 cc. methylene blue and in addition hydrogen peroxide of a final concentration varying from 0 to $M/1000$. 1 cc. of a 5 % solution of a caseinogen preparation of the oxidase was added to each tube, and the tubes were allowed to stand for 2 hours at room temperature, to allow time for the peroxide to act on the enzyme. 0·1 cc. hypoxanthine was then added to each tube, all the tubes were evacuated, and the reduction times determined as before.

The results are shown graphically in Fig. 3. It will be seen that the hydrogen peroxide in a concentration of one-millionth molar has practically doubled the activity of the enzyme, and that quite a marked effect is produced by a concentration of one-hundredmillionth molar. With concentrations of peroxide greater than $10^{-5}$ $M$ the retarding effect of the peroxide is

partly due to its reoxidation of the reduced methylene blue diminishing the apparent velocity of reduction. Since the initial concentration of the methylene blue was about $10^{-4}$ *M* this effect can introduce no appreciable error into the results with peroxide concentrations less than $10^{-5}$ *M*, but must be allowed for with concentrations greater than this. The falling part of the curve in Fig. 3 does not therefore necessarily represent an actual destruction of the enzyme in this experiment. With a weaker solution of enzyme, however, the destruction shows up clearly, even with $H_2O_2$ concentrations of the order of $10^{-6}$ *M* where the reoxidation effect is entirely negligible (*e.g.* in Exp. 11 below).

The optimum peroxide concentration for acceleration with enzyme concentrations of the order used in Exp. 9 was found in a number of experiments to be about one-millionth molar, and if the conditions are correctly adjusted, the increase of activity may become very large, as in the following experiment.

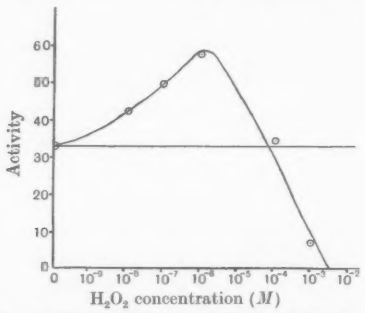


Fig. 3. Effect of varying concentrations of $H_2O_2$. The horizontal straight line represents the activity without $H_2O_2$.

*Exp.* 10. Two tubes were made up, each containing 0·5 cc. 5 % caseinogen preparation + 5 cc. buffer + 1 cc. methylene blue and the amount of peroxide given. The tubes were allowed to stand for 1½ hours and the reduction-times with hypoxanthine then determined.

| Amount of $H_2O_2$ | Reduction time at 15° |
|---|---|
| 0 | 61′ |
| $10^{-6}$ *M* | 24′ |

The reversal of the effect by changing the enzyme concentration which was found in Exps. 6 and 7 was also obtained when using hydrogen peroxide instead of the light, as follows.

*Exp.* 11. Four tubes were prepared, each containing 5 cc. buffer + 1 cc. methylene blue. To one pair of the tubes 0·2 cc. 5 % caseinogen preparation was added, and to the other pair 0·5 cc. of the same solution. One tube of each pair contained $10^{-6}$ *M* hydrogen peroxide. The tubes were allowed to stand for 1½ hours before the activities with hypoxanthine were tested.

| | Reduction time at 15° | |
|---|---|---|
| Amount of oxidase | Without $H_2O_2$ | With $H_2O_2$ |
| 0·2 cc. | 170′ | 195′ |
| 0·5 cc. | 84′ | 70′ |

The necessity for allowing sufficient time for the peroxide to act upon the enzyme is shown by the following experiment, in which the peroxide was allowed to act for varying periods of time before the activity was tested.

*Exp.* 12. Each tube contained 0·5 cc. 5 % caseinogen preparation + 5 cc. buffer + 1 cc. methylene blue + hydrogen peroxide in a concentration of $10^{-6}$ *M*. The tubes were allowed to stand for varying times before the hypoxanthine was added and the reduction time determined. The results are given in Fig. 4.

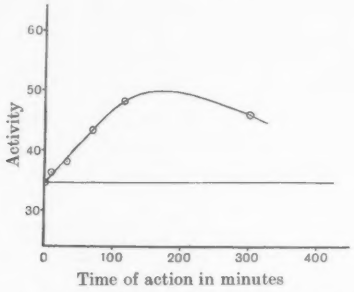


Fig. 4. Time curve of action of $10^{-6}$ *M* $H_2O_2$ on oxidase. The horizontal straight line represents the activity without $H_2O_2$.

In certain cases when using milk as the source of oxidase we have been unable to obtain effects with either hydrogen peroxide or irradiation. This we ascribe to the presence of catalase in these milks in amounts sufficient to prevent oxidation by the peroxide. Using the enzyme preparation which is free from catalase, on the other hand, we have never failed to obtain the effects, and we consider that the difference affords further support to the view that the irradiation effects are due to the formation of peroxide. It should be mentioned that curves exactly similar to that of Fig. 3 have been obtained with certain milks, which were presumably deficient in catalase.

Aldehydes are capable of replacing hypoxanthine in the oxidase system as the hydrogen donator, and nitrates can be used instead of methylene blue as the oxidiser in the reaction. The activity of the enzyme was therefore compared before and after the action of peroxide, using these substances as the reactants.

*Exp.* 13. The tubes contained 0·5 cc. 5 % caseinogen solution + 5 cc. buffer + 1 cc. methylene blue or 5 % $NaNO_3$, with in addition $10^{-6}$ *M* $H_2O_2$ in two of the tubes. They were allowed to stand 1 hour. Then 0·5 cc. 1 % acetaldehyde was added to each tube, instead of the usual hypoxanthine, and all the tubes immediately evacuated. In the case of the tubes containing methylene blue the reduction times were determined in the usual way. The tubes containing nitrate were kept at 15° for 2 hours; and they were then opened and the Griess-Ilosvay reagent was added to determine the amount of nitrate which had been reduced to nitrite.

| Amount of $H_2O_2$ | Reduction time methylene blue at 15° | Amount of nitrite formed |
|---|---|---|
| 0 | 105′ | ++ |
| $10^{-6}$ *M* | 85′ | +++ |

The acceleration by small amounts of peroxide still occurs when aldehyde is substituted for hypoxanthine and nitrate for methylene blue, and this affords further support to the view that its action is on the enzyme itself and not on the reactants.

The photo-oxidation of the enzyme is unaffected by the addition of cyanide, so that it is presumably not dependent on the presence of traces of catalytic metals.

*Exp.* 14. In each of four tubes was placed 5 cc. 2% solution of a whey preparation of the oxidase + 1 cc. buffer + 1 cc. methylene blue. One pair of the tubes contained in addition potassium cyanide in a concentration of *M*/400. 0·1 cc. hypoxanthine was added to each tube and the tubes were exhausted to 10 cm. pressure (cf. Exp. 1). One tube of each pair was placed in the sunlight, the others in the dark.

| | Reduction time at 15° | |
|---|---|---|
| | In light | In dark |
| Without KCN | 10′ | >50′ |
| With KCN | 10′ | >50′ |

To sum up then, we consider that the foregoing experiments show that the mechanism of the action of light on the system is as follows. An active oxidising agent, very probably hydrogen peroxide, is formed from molecular oxygen under the influence of the light in the presence of the dye, or, to a lesser extent, in the presence of the enzyme preparation[1]. This agent then acts upon the enzyme, producing first an increase and then a decrease of activity. We are at present unable to state how the increase of activity is brought about. It is possible that fresh active centres are formed from inactive precursor groups by the oxidation. It is also not impossible that the peroxide might destroy by oxidation some inhibitor substance associated with the enzyme, and so liberate fresh active centres. It would clearly be a difficult matter to decide experimentally between these hypotheses.

A number of facts in the literature are in harmony with the view given above. Straub [1904] showed that if eosin were irradiated in the presence of oxygen it would liberate iodine from potassium iodide, but that this only happened if oxygen were present. Downes and Blunt [1877] and Tappeiner and Jodlbauer [1905] showed that light was fatal to certain bacteria in the presence of oxygen, but not in its absence. Ledoux-Lebard [1902] found that eosin previously irradiated in the presence of oxygen killed paramoecia but that eosin irradiated in the absence of oxygen was harmless. All these effects might be expected if $H_2O_2$ were formed as we have supposed.

It should be pointed out that the formation of $H_2O_2$ by irradiation has no connection with the $H_2O_2$ formation shown by Thurlow [1925] to occur

[1] Which presumably contains some substance which acts in the same manner as the dye.

in the aerobic oxidation of hypoxanthine by the oxidase. Oppenheimer [1926] has suggested that the formation of $H_2O_2$ observed by Thurlow might in fact be due to the action of light on the enzyme preparation. Three facts show that this is not so: (*a*) Thurlow's results occur equally well in absolute darkness, as we have confirmed on several occasions; (*b*) Thurlow's peroxide formation occurs only in the presence of hypoxanthine or its equivalent, whereas the light effect occurs in the absence of hypoxanthine; (*c*) the amounts of $H_2O_2$ formed by light are infinitesimal in comparison with those formed in Thurlow's experiments.

We may contrast with the behaviour of the oxidase system that of the sulphydryl system. Dixon and Tunnicliffe [1923] showed that the reduction of methylene blue by cysteine and glutathione was accelerated considerably by light. But this system differs fundamentally from the oxidase, in that the effect of the light is not dependent on the presence of oxygen and takes place equally well no matter how carefully the tubes are freed from oxygen.

*Exp.* 16. Great care was taken to ensure that all tubes were oxygen-free. Each tube contained 1 cc. cysteine solution (containing 5 mg. of the hydrochloride, neutralised) + 3 cc. buffer + 0·5 cc. methylene blue.

| Amount of KCN present | Conditions of experiment | Reduction time |
|---|---|---|
| 0 | In sunlight | 1½′ |
| 0 | In diffuse light | 8′ |
| *M*/100 | In weak sunlight | 5′ |
| *M*/100 | In dark | 140′ |
| *M*/500 | In dim light (indoors) | 43′ |
| *M*/500 | In dark | 180′ |

The reduction times were determined at room temperature, but the three sets were not carried out at precisely the same temperature, and therefore the tubes in different pairs cannot be strictly compared. It will be seen that the effect of the light occurs also in the presence of cyanide, which prevents that part of the reaction which is due to metal catalysis [Harrison, 1927], so that the reduction is that due to the —SH group itself. The last pair of tubes shows that the system is extremely sensitive to light. Harrison found that it was difficult to obtain strictly reproducible results with metal-free sulphydryl compounds, and suggested that this might be due to varying intensity of light on different days; and these figures seem to support the view.

By a somewhat curious coincidence Kendall and Nord [1926] found that the reduction of indigo carmine by cysteine was greatly accelerated by the addition of hydrogen peroxide. The apparent analogy of this system with the xanthine oxidase in this respect is, however, a false one, since the peroxide acceleration with the cysteine does not occur with methylene blue but only with indigo carmine, and has been traced by Dixon and Tunnicliffe [1927] to the action of the peroxide on this dye producing a catalytically active product.

SUMMARY.

1. The reduction of methylene blue by the xanthine oxidase system is markedly accelerated by light, but only if traces of oxygen are present.

2. Previous exposure of the oxidase solution or the methylene blue to light (in the presence of oxygen) for short periods produces an acceleration of the reaction. Exposure for longer periods produces a destruction of the enzyme. Neither of these effects occurs if oxygen is absent during the irradiation.

3. Both these effects are due to the formation of an active oxidising agent, probably hydrogen peroxide, from the oxygen during the irradiation. This oxidises the oxidase; the first stage of the oxidation produces a marked increase in the activity of the enzyme, and the later stages destroy the enzyme.

4. Both these effects are produced by the addition of hydrogen peroxide in small quantities to the oxidase. The presence of one-millionth molar peroxide in general more than doubles the activity of the enzyme, while a very definite acceleration is produced by one-hundredmillionth molar peroxide. In higher concentrations the peroxide destroys the enzyme.

5. The nature of the effect produced by a peroxide solution of given strength depends on the amount of oxidase present. One and the same solution of peroxide may produce an acceleration with one solution of enzyme, and a retardation with another (weaker) solution. The same is true of solutions of methylene blue which have been exposed to light and oxygen.

6. The accelerating action of added hydrogen peroxide on the enzyme can also be seen when aldehyde is substituted for hypoxanthine and nitrate for methylene blue.

7. In presence of peroxidase, the peroxide formed during the aerobic irradiation of methylene blue solutions is able to carry out secondary oxidations (*e.g.* nitrites). This oxidation does not occur in the absence of either peroxidase, or oxygen, or light.

We wish to express our sincere thanks to Sir F. G. Hopkins for the interest he has taken in this work.

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# XVIII. THE REDUCTION OF NITRATES IN ANIMAL TISSUES.

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It has long been known that many animal tissues contain systems capable of reducing nitrates to nitrites. Stepanow [1902] investigated the presence of nitrite in the tissues of rabbits and dogs. By means of the Griess-Ilosvay reagent he found nitrite in the white matter of the brain, the lungs, bronchi, parotids, small intestine, medullary substance of the kidney, adrenals, testicles, and lymph glands. He found it absent from the grey matter of the brain, the liver, stomach, spleen, cortex of the kidney, muscle and blood. He showed, moreover, that certain tissues which normally contained no nitrite were able to form it when ground with nitrates; and also that if the animal had been fed on food free from nitrates no nitrite was found in any of the tissues.

More recently Lipschitz [1921] has shown that the dehydrases of frog's muscle (studied by Thunberg [1920] with the methylene blue technique) were able to reduce aromatic nitro-compounds, *e.g.* *m*-dinitrobenzene, and that the nitro-compound was simply equivalent to methylene blue as a hydrogen acceptor in these systems. Bach [1911] found that nitrate could replace methylene blue in the aldehyde oxidase system, which rapidly reduced it to nitrite; and Dixon and Thurlow [1924] showed that the same was true of the xanthine oxidase system.

In view of these facts it was thought interesting to investigate the reduction of nitrates by animal tissues, in order to determine which of the known systems could use nitrate as a hydrogen acceptor.

Muscle tissue was first investigated. Preliminary experiments showed that the ability of muscle to reduce added nitrates is by no means general, and seems to be confined to a comparatively small number of species. We have found that the muscles of ox, sheep, pig, rabbit, dog and chicken are quite unable to reduce nitrates, and positive results were only obtained with rat and guinea-pig muscle.

We found, on the other hand, that the livers of all these animals reduce nitrates very readily, and the liver appears to be the main seat of the phenomenon. Rat's liver reduces five to ten times as much nitrate in a given time per unit weight as rat's muscle.

The results of these preliminary experiments led us to choose rat's muscle and ox liver as convenient tissues for the investigation of the nature of the systems responsible for nitrate-reduction, and the results obtained with these two tissues are given in the two following sections of this paper.

### The reduction of nitrate by rat's muscle.

The first problem was to see whether any of the known dehydrase systems present in muscle could be responsible for the reduction. Experiments were carried out anaerobically in Thunberg vacuum-tubes as follows. Muscle from the hind legs of a freshly killed rat was finely minced and washed by shaking four times successively with 100 cc. distilled water. The washed muscle was squeezed as dry as possible, and portions of 0·3 g. were weighed out into the Thunberg tubes. 2 cc. phosphate buffer ($p_H$ 7·5) were added to each tube, together with solutions of the appropriate hydrogen donator and acceptor. The hydrogen donators were in all cases made up in 1 % solution in water and carefully neutralised, and 1 cc. of the solution was used. With methylene blue as the hydrogen acceptor the presence in the washed muscle of systems which could oxidise the following substances was readily demonstrated: succinic, formic, citric, acetic, lactic, fumaric, maleic, malic, and glutamic acids. It was found that the washed muscle by itself reduced methylene blue slowly, even after very thorough washing, so that in all cases simultaneous experiments with and without the hydrogen donator were carried out and the rates of reduction compared. The rate of the spontaneous reduction was however, in general, small in comparison with that induced by the hydrogen donator.

The experiments with nitrate were carried out anaerobically in a similar way. 1 cc. of 5 % sodium nitrate (a considerable excess) was added to each tube instead of the methylene blue, the tubes were incubated for 2 hours at 37°, and the nitrite formed during this period was estimated roughly colorimetrically after the addition of 2 cc. of Griess-Ilosvay reagent. As before, the tubes were in all cases compared with control tubes to which no hydrogen donator had been added.

The results of these experiments showed quite definitely that the reduction of nitrate to nitrite, which still occurred with the washed muscle, could not be referred to the action of any of the dehydrase systems mentioned above, since these were all totally unable to reduce nitrate. The amount of nitrite produced by the muscle itself was not increased in the slightest by the addition of any of the hydrogen donators mentioned, and the depth of colour given by the Griess-Ilosvay reagent was always exactly the same as in the control tubes with no added donator. We had previously observed that a solution of succinoxidase (prepared from muscle by Ohlsson's [1921] method) was unable to reduce nitrates in presence of succinate. In addition to the substances mentioned, the following possible hydrogen donators were tried with the washed muscle, also with negative results: acetaldehyde, pyruvic acid,

glucose, acetone, diethyl ketone, creatinine, lecithin, cholesterol, ammonia, and various extracts of liver. The latter were prepared by extracting liver with 2 % sodium carbonate and destroying their own power of reducing nitrate by acidification and subsequent reneutralisation or by boiling (see below) and were added to the muscle in order to see whether any of the thermo- or acid-stable hydrogen donators present in the liver would increase the nitrate-reduction. Boiled and acidified muscle broths were also added to the active muscle, but no greater reduction occurred.

Gscheidlen [1874] stated that muscle which had been exercised reduced more nitrate than unexercised muscle, but we have not found this. The gastrocnemii of the rat were dissected out, and one was placed in Ringer-Locke solution and made to contract by stimulation with induction shocks until too fatigued to respond. The exercised and the control muscles were then chopped finely, a portion of each was thoroughly washed with distilled water, and 0·3 g. of each portion incubated with 2 cc. buffer + 1 cc. 5 % sodium nitrate in Thunberg tubes as before. The results were as follows:

| | Nitrite reaction |
|---|---|
| Exercised muscle ... ... ... ... | + + |
| Washed exercised muscle ... ... ... | + + |
| Unexercised muscle ... ... ... | + + |
| Washed unexercised muscle ... ... | + + |

It was impossible to detect the slightest difference in the strength of the nitrite reaction between any of the tubes, so that it appears that the nitrate-reducing system has no direct connection with the products of muscular activity.

The significance of the results described above is discussed later.

*Properties of the nitrate-reducing system of rat's muscle.*

The system is not readily washed out with water: after 20 washings there is considerable activity left. Nor does previous treatment with ether to break up the cell-membranes cause it to come more readily into solution.

The system can however be extracted from muscle by $M/15$ disodium hydrogen phosphate solution. Rat's muscle was chopped, washed with water, ground with the alkaline phosphate solution, and filtered. The cloudy filtrate was precipitated by saturation with ammonium sulphate and again filtered. The precipitate contained the system unimpaired, and the filtrate, when added to muscle, did not increase the reduction.

Treating the muscle with acetone does not affect the reduction. Alcohol treatment decreases it. Standing at room temperature in the air gradually destroys the reducing power, probably because the system becomes oxidised.

If the incubation with nitrate is not carried out anaerobically, but in open tubes, the amount of nitrite formed is greatly diminished. This may be due to the system using oxygen in preference to nitrate, so that the two hydrogen acceptors compete; or it may be due to a coupled oxidation of the nitrite formed in the sense of Thurlow [1925].

The system is very sensitive to acid. If brought to $p_H$ 5 and back to neutrality the reduction is completely abolished. It is, however, more stable to alkali, and can be brought to $p_H$ 10 and back without affecting the reduction. The actual reduction process, however, is limited to a very narrow range of $p_H$, and occurs only between $p_H$ 6·5 and $p_H$ 8, with a maximum velocity at about 7·4.

The system is destroyed by heating to 70°, but survives exposure to 60° for 5 minutes.

These properties make it extremely probable that the system is enzymic in nature, but so far we have not been able to obtain separately catalyst and substrate.

Whether the reduction goes further than the nitrite stage has not been determined.

The system is specific for inorganic nitrates, and *m*-dinitrobenzene is not reduced. This gives us the apparently paradoxical result that while this system can reduce nitrate but not nitrobenzene, the muscle dehydrases can reduce nitrobenzene but not nitrate.

Particularly interesting is the effect of cyanide on the system. The presence of *M*/500 KCN completely prevents the reduction of nitrate by the muscle, so that even after several hours no nitrite can be detected by the very sensitive Griess-Ilosvay reagent. The system is therefore cyanide-sensitive in Warburg's sense in the same way as those systems which have been shown to depend on the action of small amounts of catalytic metals. We have no data to show whether this is the case in this system or not; but it is clear that the fact of a tissue respiration process being cyanide-sensitive is not in itself sufficient reason for assuming that the effect of the cyanide is due to the paralysis of an oxygen activator, as is often done, since the cyanide may inhibit reactions in which molecular oxygen is not concerned, as here.

### The reduction of nitrate by liver.

As in the case of muscle, it was found that washing with water did not remove the nitrate-reducing power of the tissue. Washed liver was therefore used in most of the experiments. The following procedure was found to give the most satisfactory results. Half a pound of ox liver was finely minced and shaken in a flask with an equal volume of ether in order to break down the cell-membranes and render the water-washing more effective. It was then placed in a two-litre flask and washed 15 to 20 times with water, until no more haemoglobin was washed out. During this process about half the liver was washed away. The final preparation consists of small light-brown granular masses. It can be kept indefinitely in the ice chest, reduces nitrate readily, and is very convenient to use.

On investigation it was found that the reduction of nitrate by the liver was due to two distinct factors: (*a*) a system apparently identical with the muscle system described above, and (*b*) the aldehyde (and xanthine) oxidase

of liver, which is probably identical with that of milk. This latter enzyme was shown to be capable of reducing nitrate (in the presence of its substrate) by Bach [1911] and by Dixon and Thurlow [1924]. The xanthine oxidase is absent from muscle [Morgan, 1926].

It is possible to separate the two factors. On extracting the washed liver by grinding with 2 % sodium carbonate solution, filtering through muslin, and neutralising the liquid, it is found that both the factors have passed into solution. In order to obtain the xanthine oxidase free from the other factor charcoal is added, the suspension shaken for a few moments, and the charcoal filtered off with suction. A clear light-brown solution of the oxidase is obtained. The separation depends upon the fact that the nitrate-reducing factor (*a*) is readily adsorbed by charcoal, whereas the oxidase is not [Dixon and Kodama, 1926]. To obtain the former, the sodium carbonate extract is brought to $p_H$ 10, and on reneutralising it is found that the oxidase has been completely destroyed, leaving the other factor intact.

The distinction between the nitrate-reducing system and the oxidase can also be shown by means of cyanide. The nitrate-reduction by the washed liver alone is completely inhibited, while in the presence of aldehyde that part of the reduction which is due to the oxidase is unaffected. The following is a typical experiment.

| Contents of tube | Nitrite reaction |
|---|---|
| 0·3 g. washed liver + 2 cc. buffer + 1 cc. $NaNO_2$ ... ... ... ... | + + |
| „ + „ + „ + 1 cc. 1 % aldehyde ... ... | + + + + |
| „ + „ + „ + 0·5 cc. *M*/30 KCN ... ... | − |
| „ + „ + „ + „ + 1 cc. aldehyde | + + |

On adding to the liver the possible hydrogen donators previously tested with muscle it was found that all gave entirely negative results with nitrate, with the exception of aldehyde and pyruvic acid. These two substances, and also hypoxanthine, reduced nitrate freely. The increase of reduction in presence of aldehyde and hypoxanthine is clearly due to the action of the oxidase, and was to be expected if the oxidase is the same as that in milk. Pyruvic acid, however, is quite unable to reduce nitrate, either with the oxidase alone or with the nitrate-reducing system alone; it only reduces with the liver. This reaction is in all probability to be attributed to the presence of a decarboxylase in the liver. This would convert the pyruvic acid into acetaldehyde, which would then act as a substrate for the oxidase, and so bring about the reduction. Gottschalk [1924] obtained acetaldehyde on incubating pyruvic acid with liver.

The addition of boiled or acid-treated liver broth to the active preparation of liver produced, as in the case of muscle, no increase in the nitrate-reduction.

The other properties of the nitrate-reducing system of liver are similar to those of the system in muscle. Treatment with acetone does not affect the reduction, but alcohol partially destroys it. It is thermolabile, and is destroyed by heating to 70°, but not by heating to 60° for 5 minutes. It is

acid-sensitive, and is completely destroyed at $p_H$ 5; but, on the alkaline side, $p_H$ 10 leaves it quite unaffected. It is cyanide-sensitive, as mentioned above. The similarity in properties strongly suggests that the nitrate-reducing systems in muscle and liver are identical.

Attempts have also been made to separate catalyst and substrate in the liver system by precipitation and other methods, but without success.

The washed liver contains very little glutathione, to judge from the nitroprusside reaction, but it will very readily reduce oxidised glutathione. A preparation which gives an almost negative nitroprusside test will, after having been incubated anaerobically for a short period with oxidised glutathione, give a very strong reaction. This fact may be of importance in the study of the glutathione system. This reduction is unaffected by boiling the liver, whereas of course the nitrate-reduction is completely destroyed, so that the two reductions cannot be due to the same factors.

The thoroughly washed liver is still able to reduce methylene blue, but we are as yet unable to say whether part or all of this reducing power is due to the nitrate-reducing system or not. The liver also reduces the series of dyes used by Mansfield Clark as $r_H$ indicators. The results obtained with these dyes, however, are somewhat unexpected and difficult to explain, though perfectly definite. They are as follows. The dyes were freshly made up in aqueous solution in concentrations equivalent to the 1/5000 methylene blue solution used. (The names and $r_H$ ranges of the dyes have been previously given in this *Journal* [Dixon, 1926, p. 714], and they are here referred to simply by their series-numbers. They are numbered in ascending $r_H$ order, so that No. 9 is theoretically the easiest and No. 1 the most difficult to reduce. No. 3 *a* is methylene blue.) The reductions were carried out in Thunberg tubes as before, and 0·3 g. of thoroughly washed liver + 2 cc. buffer + 1 cc. of the dye were used in each tube. The table gives the reduction-times (in minutes) with and without the addition of 1 cc. 1 % acetaldehyde.

| Dye No. | Reduction-time | | Dye No. | Reduction-time | |
|---|---|---|---|---|---|
| | Alone | +aldehyde | | Alone | +aldehyde |
| 1 | 120 | 70 | 5 | 45 | 50 |
| 2 | 420 | 100 | 6 | 17 | 35 |
| 3 | 480 | 100 | 7 | 20 | 38 |
| 3 *a* | 60 | 40 | 8 | 25 | 54 |
| 4 | 45 | 50 | 9 | 8 | 25 |

It will be seen that while the first four dyes behave normally in being reduced more rapidly in the presence of aldehyde than in its absence, reduction of the last six dyes is actually *slowed* by the aldehyde. The cause of this is not clear, but it is possible that there is some active reducing system in the liver whose potential is such that it can reduce dyes 4 to 9, but cannot reduce dyes 1 to 3 *a*. If now this system is inhibited by the presence of aldehyde, results similar to those found will be produced. For, in the case of the last six dyes, where the reduction may be mainly due to this system, the inhibition produced by the action of the aldehyde on the system may

obscure the acceleration produced by the action of the aldehyde oxidase. With the first four dyes, on the other hand, the system plays no part, and consequently the action of the oxidase shows up. This is merely a suggestion, but it is not easy at the moment to see any other explanation.

## Discussion.

Though the significance of some of the facts given in this paper is not yet clear, a number of interesting points are brought out by the work.

It appears fairly certain that the nitrate-reducing system in muscle, and the corresponding system in liver, cannot be identical with any of the hitherto described reducing enzymes, and it seems that we must attribute the reduction to a new enzyme system. As to its significance in connection with tissue respiration processes we can of course say little as yet.

As we have pointed out above, the system is very active in the livers of all the animal species examined; but the fact that it is absent from the muscles of all but two of the species affords another example of the curious and apparently haphazard distribution of some of the systems concerned in biological oxidations (compare the xanthine oxidase [Morgan, 1926]).

The fact that the Thunberg tissue dehydrases are unable to reduce nitrate is a fact of considerable importance in considering the ability of nitrate to act as a direct hydrogen acceptor. Dixon and Thurlow, working on the milk oxidase, believed that nitrate was simply equivalent to methylene blue as a hydrogen acceptor, and it is possible that this is the case in the system with which they were dealing. It is clear, however, that the two substances are by no means always equivalent.

We have not sufficient data as yet to decide whether the failure of nitrate to replace methylene blue in the Thunberg dehydrase systems is due to an insufficiently high reducing power in these enzyme systems, to a difference in the accessibility of the two substances to the active enzyme surface, or to the lack of a nitrate-activating catalyst of the type studied by Quastel, Stephenson and Whetham [1925]. Each of these possibilities would seem to provide an adequate explanation.

The first possibility involves the assumption that methylene blue is more easily reduced than nitrate, and that the dehydrases, with the exception of the aldehyde and xanthine oxidase, have reducing powers greater than that necessary to reduce methylene blue but less than that necessary to reduce nitrate. The xanthine oxidase seems to be a particularly strongly reducing system compared with the other dehydrases. Its reducing power must be much higher than that of the succinoxidase for instance, for while the latter system comes to an equilibrium with methylene blue considerably before the dye is completely reduced [Thunberg, 1925], the xanthine oxidase reduces methylene blue completely (*i.e.* more than 99·9999 %), as shown by Clark [1927]. Although a number of systems are known which will reduce methylene

blue but not nitrate, we are not aware of any[1] which will reduce nitrate but not methylene blue.

The possibility that the difference in behaviour is due to a difference in accessibility must be borne in mind; but, although it may explain the facts in the systems under consideration, it cannot account for the difference in behaviour with homogeneous systems (*e.g.* cysteine, referred to below).

In connection with the third possibility it should be pointed out that, while it seems probable that the muscle dehydrases may be able to reduce nitrate only when the latter is activated by a catalyst, it seems quite possible nevertheless that nitrate may be directly reduced without activation in such systems as the xanthine oxidase. Quastel, Stephenson and Whetham used as a test of nitrate-activation the ability of the activated nitrate to oxidise reduced methylene blue, which nitrate by itself is unable to do. We have found that using this test no nitrate-activation can be detected in the xanthine oxidase system: in one limb of a branched vacuum-tube was placed sodium nitrate solution, and in the other limb xanthine oxidase solution in phosphate buffer together with methylene blue and an amount of hypoxanthine just sufficient to reduce the latter; after careful evacuation of the tube and allowing to stand until the methylene blue was reduced, the nitrate solution was immediately run into this solution by tilting the tube. Not the slightest trace of blue colour appeared. If then the oxidation of leuco-methylene blue is a reliable indication of the presence of activated nitrate it would seem that the xanthine oxidase system does not contain a nitrate-activating catalyst and that the oxidase is able to reduce nitrate directly. If the nitrate is added initially with the methylene blue the reduction of the latter is slowed, presumably because the nitrate competes with the methylene blue as a hydrogen acceptor.

If it is assumed that nitrate invariably requires activation before it can be reduced in the tissues it is clear that the catalyst responsible must be associated in a very special way with particular oxidase systems in the cell. For example, the succinic oxidase in, say, a liver extract cannot reduce nitrate, while *in the same solution* the aldehyde oxidase reduces nitrate freely, so that the activator, if present, must be in intimate association with the latter system in such a way that its action is not available for other systems. The same applies also to the nitrate-reducing system in muscle.

Nitrates differ from methylene blue in giving no oxidation-potential at a platinum electrode, in this respect resembling the aromatic nitro-compounds. The latter are nevertheless easily reduced, both *in vitro*, for example by —SH compounds (cysteine, glutathione, etc.), and also by the dehydrases. Nitrates are not reduced by cysteine, unless a nitrate-activating catalyst is present (Quastel, Stephenson and Whetham). They are, however, reduced *in vitro* by such reducing systems as zinc dust suspended in *neutral* solution—a system which has reducing properties which very closely parallel those of the xanthine

[1] One of us (F. B.) will deal with the aldehyde oxidase of the potato in a paper to be published shortly.

oxidase system. For instance, in addition to the reduction of the usual dyes, etc. they both take up molecular oxygen, forming hydrogen peroxide, which is subsequently reduced to water; the oxygen uptake is not prevented by cyanide; they both reduce nitrates and aromatic nitro-compounds; and they both fail to reduce the —SS— group[1]. Other points of similarity might be mentioned. The Thunberg dehydrases, on the other hand, would seem rather to correspond in reducing properties with —SH compounds. They fail to reduce nitrates in the absence of a special catalyst; the oxidation of the —SH group by molecular oxygen is abolished by small amounts of cyanide [Warburg and Sakuma, 1923; Harrison, 1924], which is true also of, at any rate, the oxidation of succinic acid by the succinoxidase [Fleisch, 1924]; and both —SH compounds and the succinoxidase system reduce oxygen some hundreds of times faster than methylene blue, which is not the case with the xanthine oxidase.

We would suggest provisionally that, while the majority of the dehydrases can reduce nitrate only in the presence of a special catalyst, certain of them are able to reduce nitrate directly in the absence of such a catalyst.

## Summary.

1. While the livers of all the animals studied reduce nitrate freely, nitrate-reduction in muscle is confined to the rat and the guinea-pig. A study of nitrate-reduction in rat's muscle and ox liver has been made.

2. The reduction of nitrate by muscle cannot be referred to any of the known reducing systems present. The dehydrases of muscle are incapable of reducing nitrate. No hydrogen donator has been found which will increase the reduction of nitrate by washed muscle.

3. The properties of the nitrate-reducing system are described. It appears to be enzymic in nature.

4. The nitrate-reduction is completely abolished by the addition of $M/500$ cyanide.

5. In liver the nitrate-reduction is due to two factors: (*a*) a system having the same properties as that in muscle, and (*b*) the aldehyde (xanthine) oxidase, probably identical with that in milk. These two factors can be separated. No hydrogen donator, other than the substrates of the oxidase, has been found which will increase the reduction of nitrate by liver.

6. These facts are discussed in relation to theories of biological oxidation processes.

We wish to thank Sir F. G. Hopkins for his interest and advice.

[1] Zinc dust in acid solution of course reduces the —SS— group.

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# XIX. THE QUANTITATIVE DETERMINATION OF VITAMIN D.

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The ordinary procedure for the quantitative determination of vitamins consists in giving the experimental animals during a preparatory period a diet which is complete in every respect, with the one exception that it lacks the vitamin with which the experiment is concerned. As soon as characteristic deficiency symptoms have definitely developed, the test period is initiated, during which the minimum curative dose is determined. As is already well known, we have in the case of vitamin C to determine the protective dose, for the condition of the teeth in scorbutic animals interferes with their development.

Certain difficulties are encountered in the quantitative determination of vitamin D which has of late years attracted much attention. The first requirement is that the animals (rats) shall develop rickets during the preparatory period. Very suggestive evidence of this condition are certain peculiarities in the animals' gait and movements, but these clinical signs do not warrant definite conclusions as to the degree which the disease has reached, and still less do they indicate the stages in a recovery which may be taking place. For the changes which occur in response to the administration of the missing vitamin are so gradual that the degree of the improvement is difficult to estimate.

Definite information can, of course, be obtained by a post-mortem examination. There are also the well-known analytical methods, such for example, as the determination of the water, ash and calcium, or of the ratio of the inorganic to the organic material in the long bones. These methods require considerable time and care; the bones have first to be most carefully freed from the soft tissues, and have then to be dried and analysed. Considerable simplification has been achieved by McCollum and his fellow-workers with their delicate and much-used line test. This renders possible a quantitative determination both of the severity of the rickets, and of the progress of recovery should this occur. Lastly, we may mention the reaction of the faeces [Zucker and Barnett, 1923; Jephcott and Bacharach, 1926] which

normally are acid, but which under a high calcium, low phosphorus rickets-producing diet become alkaline, turning acid again when an antirachitic diet is given. It is, however, not known whether changes in the $p_H$ are proportional to the severity of the disease or not. In the most recent publication [Willimott and Wokes, 1927] the opinion is held that the alkaline reaction of the faeces is specifically indicative of absence of vitamin D, whether rickets do or do not develop.

At the end of the preparatory period, *i.e.* at the beginning of the test period, the quantitative diagnosis of rickets is thus made by chemical analysis or the line test, supported by the anatomical examination[1].

A fault common to all these methods is, however, that the same animals are not employed for the preparatory and the test periods. One is obliged to proceed in such a way that a certain number of animals are put on a rachitogenic diet and some of them are, after a suitable interval, killed and examined. If all are found to be rachitic, it is assumed that the survivors are so also. This procedure, as will shortly be pointed out, entails the possibility of a serious error which cannot be evaded even if some of the animals are kept as pathological controls.

The first condition for a uniform reaction is that the breeding stock as well as the lactating mothers and their progeny should have a diet which is both suitable and of a constant composition. The importance of this condition can be realised by a study of, among others, the exhaustive researches which have recently been published from the Lister Institute [Chick, 1926; Smith and Chick, 1926; Chick and Roscoe, 1926]. It was observed that the difference between winter and summer milk was sufficient to cause fluctuations in the reactions, the greater content of vitamin D in summer milk conferring greater resistance to a rachitogenic diet in the summer than in the winter. This difficulty was avoided by the use of dried winter milk in the summer. But, as Chick's tables [see 1926, p. 29] show, all the fluctuations were not abolished by this procedure. Of 10 young rats whose mothers had been given the dried winter milk, two were found to have long bones with a calcium content (dry weight) of 9·8 and 9·9 % respectively, whereas the rest showed higher figures, the percentage in the case of one animal being as high as 15·4. When such variations occur in tests carried out by experts, the only conclusion to be drawn is that these variations depend on fundamental, individual differences.

The existence of such differences has been confirmed by us by numerous tests. They may at any rate appear under a diet (Steenbock and Black, 2965) employed by us, either as familial (one litter differing from another) or as individual, half the number of animals in one litter, for example, developing well-defined rickets during the preparatory period, while others of the same

[1] The determination of the phosphorus content of the blood by micro-analysis is very useful when applied to large animals, but can hardly be used in connection with young rats, which have only a few cubic centimetres of blood.

litter remain nearly free from rickets, or show only early signs of it. These individual variations are most striking, notably when the litters are limited, as is our usual practice, to six or seven, so that all shall receive sufficient milk from the mother.

Similar variations recurred continually among a great number of litters which included a total of about 1000 young rats. Yet the extraneous conditions seemed to have been perfectly identical for all. Their parents had, throughout several generations, 5 to 6 years, lived under very uniform conditions—the same diet, the same quantity of light, the same opportunity for exercise, and so on. In spite of this, however, it was observed that, for example, of 30 animals on a rachitogenic diet, about 80 % showed well-developed rickets after 3 to 4 weeks, whereas the remainder did not suffer from rickets or only to a slight extent. Had 5 animals been killed and examined, they might well have been among the 80 %. One would, in such a case, have had in the test groups many almost normal animals, and incorrect conclusions might have been drawn with regard to the efficiency of the antirachitic substance tested. We have repeatedly let such resistant animals continue for an extra week on the rachitogenic diet without the development of any serious changes.

It may be of interest to remember that the human being is also subject to similar, very puzzling variations in susceptibility to rickets. On one and the same diet, and even on the milk of the same wet-nurse, it is not rare to find that one infant becomes rachitic and another not. Still more striking is the observation that twins are not the same with regard to rickets, although it would be impossible to have more uniform conditions than those under which they live. They have the same mother and father, and have lived in the same uterus for 9 months. Sex also plays a part. In Priestley's copious statistics, dealing with more than 75,000 cases of rickets, this disease was twice as common among boys as among girls [quoted by Pfaundler, 1927]. We have not made any observations as to sex incidence of the disease in rats.

We must, therefore, conclude that, in animals as well as in human beings, susceptibility to rickets depends on certain unknown factors which play a part in spite of uniform extraneous conditions, a uniform diet, etc., which do not eliminate these factors. The consequent mistakes can only be avoided by the exclusive use of animals with definitely demonstrable rickets, and by *employing the same animals for the preparatory and the test periods*, as is done with determinations of vitamins A and B. The radiographic observations made by many workers from time to time constitute a sure and logical way out of the difficulty. Many, including the present author, have been apt to regard this process as complicated and difficult. On closer study, it has proved to be easy and simple, and in our experience it provides a reliable, quantitative means of estimating both the degree of the disease and the changes provoked by the administration of an antirachitic substance. The following is a description of the technique we have employed.

The breeding stock employed has for several years lived during summer and winter at an approximately uniform temperature, about 21–22°, in a room facing north and provided with ordinary window glass. The dietary consisted originally of bread, yellow whole maize and whole milk *ad libitum*. Twice a week fresh vegetables (carrots, turnips and cabbages) and boiled meat were given. When we suspected that the young rats were getting too large a reserve supply of vitamins A and D from the milk of this dietary, we have, during the past year, mixed the milk with equal quantities of water, and we have been satisfied with this change. We have not encountered the difficulties, depending on the seasonal variations in the milk, described by the authors from the Lister Institute to whom we have referred above. Observations made on butter at different seasons of the year have shown us that, at any rate with regard to vitamin A, and probably also with regard to vitamin D, there is not such a great difference between summer milk and winter milk in Norway as there is in other countries. The explanation is probably to be found in the fact that roots and seeds are used less in the winter in Norway than elsewhere. The cows' winter fodder consists principally of hay, much of which comes from mountain levels favoured by active and prolonged insolation. Even as far south as Oslo, daylight during June and July lasts about 18 hours, the sun rising between 3 and 4 a.m., and setting between 9 and 10 p.m. Yet another factor acting in the same direction is the extensive use during the winter of the herring meal or cake, which contains vitamins A and D.

The young rats are taken from the mother when about 24 days old, and are put on a rachitogenic diet when they weigh 45–50 g. (the first weighing). After we had tested several dietaries, we have continued to use, for the time being, No. 2965 of Steenbock and Black [1925]. This consists of yellow corn 76 %, wheat gluten 20 %, calcium carbonate 3 % and sodium chloride 1 %, or McCollum's modification of the same diet (25 parts of maize replaced by 25 parts of whole wheat—information which we owe to the courtesy of E. M. Houghton). We have not been able to observe any definite difference in the action of these two dietaries. The animals develop well on both, and at the worst show naked eye signs only of commencing xerophthalmia.

After the animals have been about 25 days on the rachitogenic diet, they are weighed for the second time, and then a skiagram is taken of the left knee-joint which is chosen in every case for the sake of uniformity (a long series of comparative observations showed that the two knee-joints were always involved to the same extent). The rat is anaesthetised in a large tumbler into which a few cc. of ether are poured. The anaesthesia must be light to avoid the risk of being fatal. It is maintained, when necessary, by the use of a little funnel-shaped mask on to which some drops of ether are dripped. The rat is then immobilised, the left knee being gently extended. With practice, the taking and the developing of the photograph is very easy and does not require much time. A skilled laboratory assistant takes and

develops 20 films in 2 to 3 hours. Thus, a very great and welcome economy of time and labour is effected, as compared with the time and labour needed for the line test, not to mention the chemical analysis. When the small dental films measuring 3 by 4 cm. are used, the cost is very moderate. (We have used Eastman Duplicitised X-ray films.)

*The test period.*

The time needed for an antirachitic substance, such as cod-liver oil, to make its action demonstrable, depends on the dosage. Large doses act much more rapidly than smaller doses. We use 3 mg. of cod-liver oil a day as the dosage from which we start. After exactly 6 days, the rats are weighed for the third time. It is important that their growth should be satisfactory, for, as is well known, starvation helps to deposit calcium in the metaphysis and effects improvement in the rickets (cf. the modern treatment of rickets in human beings by a moderate underfeeding). We might also weigh the daily food intake, but this would entail giving each animal a separate cage and complicating the work to an unnecessary degree. Throughout the test period, the rats are, of course, still kept on the rachitogenic dietary, and if they continue to develop just as well as during the preparatory period, any improvement which occurs must be traced only to the antirachitic substance added to the dietary. If cod-liver oil is the subject of the test, growth is often promoted, and thus a further assurance is given that the positive results are correctly interpreted. Rats, which during the test period remain at the same weight or lose weight, are discarded.

Both during the preparatory and the test periods, the rats are kept in lots of four each in large cages which permit of exercise. The floor is covered with sawdust, and the rats have, therefore, free access to faeces.

*Estimation of the results.*

The degree of the rickets is indicated by one to four crosses. Thus, + + + + indicate a 4 to 5 mm.-wide, uncalcified, or almost uncalcified (see below) metaphysis which lies between the diaphysis and epiphysis of the tibia. A corresponding picture is also to be seen in the distal end of the femur but is less clear. A slightly narrower metaphysis, *e.g.* one of 3 to 4 mm. is indicated by + + +. Occasionally the transition from diaphysis to metaphysis is not quite sharply defined, or a narrow calcified strip, lying just over the proximal end of the diaphysis, is visible. In such cases the degree of the rickets is put at a lower figure, *e.g.* in class 3 instead of in class 4. Narrower metaphyses, with or without calcified strips, are indicated by + + and +. The metaphysis is often almost rectangular, but in the slighter degrees of rickets its shape is that of a blunt-cornered triangle, the base of which faces the fibula. The more advanced the disease, the more clearly do changes in the bones appear. In every group of four rats it is, therefore, necessary that at least two of them suffer from + + + + or + + + rickets. The different

stages in the disease are indicated in Figs. 1 to 4 (Plate I) which are obtained by first taking a diapositive of the film and then copying it. In this way the same distribution of light and shade is obtained as in the original negative.

The action of the substance tested is judged by comparing the pictures taken at the beginning and end of the test period. The sign ÷ indicates that the disease has become worse, and the sign ± that no change has occurred, while recovery is indicated by one to four crosses. It is easy to decide whether the substance tested is, or is not, potent. The quantitative evaluation of a recovery involves, of course, a certain subjective element, but with practice it can be done with satisfactory regularity. Commencement of recovery is indicated, as a rule, by a somewhat ill-defined calcification shadow (a light strip on the film) in the proximal portion of the metaphysis, separated from the epiphysis by an uncalcified zone which appears black on the film. The commencing calcification often fails to extend throughout the whole of the horizontal width of the metaphysis, there being a gap in the middle. As recovery proceeds, the calcified strip becomes wider, a couple of zones of calcification may appear, and finally the whole of the metaphysis shows calcification, and the picture is practically normal.

The efficacy of the substance tested is indicated in units per g. on a principle analogous to that adopted by the American pharmacopoeia in its prescriptions for vitamin A. Thus, if a daily dose of 2 mg. yields a positive result, the substance contains 500 vitamin D units per g. etc.

*Examples.*

Lot 225. Cod-liver oil 346. Daily dose 4 mg.

| Rat No. | Rickets | Healing | Change in wt during the test period g. |
|---|---|---|---|
| 1192 | + + + | + + + + | +14 |
| 1195 | + + + | + + + | +10 |
| 1200 | + + + | + + + + | + 7 |
| 1204 | + + + | + + + | + 9 |

*Result.* Very great antirachitic action, together with great gain of weight.

*Conclusion.* New test with a smaller dose, *e.g.* 2 mg.

Lot 296. Cod-liver oil 386. Daily dose 3 mg.

| | | | |
|---|---|---|---|
| 1860 | + + + | + + | +5 |
| 1866 | + + + | + + + | +4 |
| 1871 | + + | + + + | +6 |
| 1880 | + + + | + + + | +1 |

*Conclusion.* About 300 vitamin D units per g.

## Summary.

A quantitative method for determining vitamin D is described. Its chief features are the following.

(1) The same rats are used for the preparatory period as for the test period.

Fig. 1.
Rickets
+

Fig. 2.
Rickets
+ +

Fig. 3.
Rickets
+ + +

Fig. 4.
Rickets
+ + + +

(2) *Preparatory period.* The rats are put on a Steenbock and Black diet 2965, or a similar diet. After about 25 days a skiagram of the left knee-joint is taken, and the rats are again weighed.

(3) *Test period.* The antirachitic substance to be tested is given for 6 days, after which another skiagram is taken and the rats are again weighed.

(4) The efficacy is indicated in units per g. on a principle analogous to that adopted by the United States Pharmacopoeia in its prescription for vitamin A.

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# XX. THE CHEMICAL CHANGES TAKING PLACE IN THE PROTEINS OF MUSCULAR TISSUE WHEN PASSING INTO RIGOR.

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In earlier papers it has been shown that gelatin, after treatment with even relatively dilute acids, yields a larger amount of the diamino-nitrogen fraction (precipitable by phosphotungstic acid) in the hydrolysis products than does untreated gelatin [Knaggs, 1923; Schryver and Buston, 1927; Thornley, 1927].

It was thought that the lactic acid produced during the contraction of muscular tissue might cause changes in the proteins of that tissue similar to those produced when gelatin is treated with acid. This was actually found to be the case. The experiments were carried out with the gastrocnemius and sartorius muscles of frogs. The hydrolyses were carried out by the micro-method of Thimann [1926]. By this method, the Hausmann numbers of the proteins could be determined in duplicate with small muscles of about 0·3 g. weight. The hydrolysis was always started by throwing the muscle into about four times its weight of boiling 20 % hydrochloric acid. When the muscle was thrown into acid immediately after excision from the animal, it gave a single twitch when coming into contact with the hot vapour or liquid. Under these conditions, about 30 % of diamino-nitrogen was obtained. If similar muscles were allowed to pass into rigor by remaining in a moist chamber for about 20 minutes at 40°, the diamino-nitrogen rose to about 36 %. The preliminary twitch could, however, be completely abolished by a previous treatment of the animal with either urethane or $\beta$-eucaine. Under these circumstances, the diamino-nitrogen of the muscles after hydrolysis sank to 26–27 %. If the muscles were allowed to pass out of rigor by standing for several hours in a moist chamber, the diamino-nitrogen sank from about 36 % (the number obtained for complete rigor) to about 30–31 %. The results are very analogous to the results obtained by the treatment of gelatin with acids.

*Experimental details.*

*Frog I.*

| | Muscles hydrolysed directly after extirpation | | Muscles allowed to pass into rigor by standing in moist chamber for 20 minutes at 40° | |
|---|---|---|---|---|
| | l. gastrocnemius | l. sartorius | r. gastrocnemius | r. sartorius |
| Amide-N | 7·8 % | 8·0 % | 7·7 % | 7·6 % |
| Humin-N | 1·5 | 1·4 | 1·3 | 1·3 |
| Diamino-N | 29·6 | 29·7 | 35·5 | 35·5 |
| Other N | 60·8 | 60·9 | 56·0 | 56·0 |

*Frog II.* 1 cc. of urethane (in 0·5 % solution) was injected into the lymph spaces before extirpation of muscles.

| | l. gastrocnemius | l. sartorius | r. gastrocnemius | r. sartorius |
|---|---|---|---|---|
| Amide-N | 8·2 % | 8·2 % | 8·2 % | 7·9 % |
| Humin-N | 1·6 | 1·6 | 1·5 | 1·5 |
| Diamino-N | 25·9 | 25·8 | 26·0 | 25·9 |
| Other N | 63·8 | 63·9 | 64·1 | 64·2 |

*Frog III.* 2 cc. of 1 % β-eucaine solution was injected into lymph spaces before extirpation of muscle.

| | l. gastrocnemius | l. sartorius | r. gastrocnemius | r. sartorius |
|---|---|---|---|---|
| Amide-N | 7·9 % | 7·3 % | 8·0 % | 7·5 % |
| Humin-N | 1·5 | 1·3 | 1·5 | 1·4 |
| Diamino-N | 26·4 | 26·7 | 26·1 | — |
| Other N | 64·3 | 64·7 | 64·7 | 64·5 |

*Frog IV.* One pair of muscles was allowed to go into rigor by keeping at 40° for 20 minutes. In the other pair, rigor was allowed to pass off by keeping the muscles at 40° for 4 hours.

| | Muscles in rigor | | Muscles after disappearance of rigor | |
|---|---|---|---|---|
| | gastrocnemius | sartorius | gastrocnemius | sartorius |
| Amide-N | 7·2 % | 7·4 % | 7·5 % | 7·2 % |
| Humin-N | 1·4 | 1·5 | 1·6 | 1·6 |
| Diamino-N | 36·0 | 35·5 | 31·2 | 31·1 |
| Other N | 55·3 | 55·6 | 59·8 | 60·0 |

*Frog V.* Muscles hydrolysed directly after extirpation.

| | gastrocnemius | sartorius |
|---|---|---|
| Amide-N | 7·8 % | 7·3 % |
| Humin-N | 1·5 | 1·5 |
| Diamino-N | 30·05 | 30·2 |
| Other N | 60·6 | 60·8 |

*Frog VI.* Muscles kept in moist chamber at 40° till rigor had completely passed off.

| | gastrocnemius | sartorius |
|---|---|---|
| Amide-N | 7·5 % | 7·6 % |
| Humin-N | 1·5 | 1·4 |
| Diamino-N | 30·8 | 30·6 |
| Other N | 60·1 | 60·2 |

All the above numbers are averages of two closely agreeing analyses. It will be observed that the amide-nitrogen varies only within narrow limits, whereas the diamino-nitrogen shows wide variations according to the state of the muscles.

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# XXI. THE CHEMICAL COMPOSITION OF THE MILK OF COWS RECEIVING COD-LIVER OIL.

## (PRELIMINARY PAPER.)

By ELFRIDA CONSTANCE VICTORIA MATTICK.

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(*Received December 29th, 1927.*)

Since the butter from the milk of cows fed on cod-liver oil was found to be much higher in antirachitic properties than that from cows on ordinary diet [see Golding and Zilva, 1928], and since rickets is a calcium deficiency disease, it was thought that if any of the mineral salts were affected by the feeding of cod-liver oil the most probable ones would be the calcium salts, and that it was therefore desirable to estimate the amounts of the total and diffusible calcium present in the milk of cows receiving cod-liver oil in comparison with that of cows on a normal diet.

### *General husbandry.*

The experiments were carried out over a period of five months, from January until July 1927 and the details of management of the cows is given elsewhere [Golding and Zilva, 1928]. The four cows from which samples were taken were Flora I and Winsome II, which both received cod-liver oil, and Flora II and Clara, who received similar rations, *i.e.* hay, silage, mangolds and concentrates, but in lieu of cod-liver oil were given small quantities of arachis (pea-nut) oil.

From January 18 until April 22, the cows remained in the byre and covered yard; from April 22 until June 14 they were out at grass during the day time, but were brought in at night; from June 14 onwards they remained out both day and night.

### *Quantities of cod-liver oil.*

The quantities of cod-liver oil and arachis oil fed daily were as follows:

| | | | |
|---|---|---|---|
| *To Flora I:* | 2 oz. | cod-liver oil | from Jan. 6–11 |
| | 4 | ,, | ,, 11–29 |
| | 6 | ,, | ,, 29–Feb. 26 |
| | 8 | ,, | Feb. 26–May 3 |
| | 7 | ,, | May 3– 6 |
| | 6 | ,, | ,, 6–10 |
| | 5 | ,, | ,, 10–16 |
| | 4 | ,, | ,, 16–July 6 |

*To Winsome II:* ½ oz. cod-liver oil from Jan. 6– 8
2 „ „ „ 8–11
4 „ „ „ 11–29
6 „ „ „ 29–May 3
5 „ „ May 3–16
4 „ „ „ 16–June 7
2 „ „ June 7–July 17

*To Flora II:* 2 oz. arachis oil from Feb. 12–24
4 „ „ „ 24–Mar. 5
6 „ „ Mar. 5–13
8 „ „ „ 13–28

*To Clara:* 2 oz. arachis oil from Feb. 12–24
4 „ „ „ 24–Mar. 6
6 „ „ Mar. 6–13
8 „ „ „ 13–30
2 oz. cod-liver oil from May 4 onward.

*Chemical analysis.*

The following estimations were made.

(1) *Titratable acidity* by means of $N/9$ NaOH.

(2) *Time of coagulation by rennet.* The method for the determination is one which has been in use in this laboratory for several years and is known to give consistent results. 250 cc. of milk are coagulated in an insulated metal vessel with 3 cc. of rennet, and the time of coagulation, measured in minutes and seconds by means of a stop watch, is taken from the moment when the rennet is added until coagulation begins.

The base of the insulated vessel is fitted with an agate nozzle with an opening of 1 mm. in diameter through which the milk flows until coagulation takes place.

It is hoped to publish shortly more details of this method and the nature of the results obtained.

(3) *Total calcium.* The total calcium was precipitated as oxalate from the ash of a weighed quantity of milk (approx. 25 g.), converted into sulphate and the liberated oxalic acid titrated with $N/10$ permanganate.

(4) *Diffusible calcium.* To determine the amount of diffusible calcium, 25 cc. of milk were dialysed against 150 cc. of 3 % saline for 18 hours. At the end of this time the calcium in the diffusate was estimated by the method described under total calcium, except that the potassium permanganate used was $N/50$. The determinations were made in duplicate and corrections were applied for variations in the porosity of the capsules.

(5) *Total phosphorus.* This was also estimated on the ash of a known weight of milk. The phosphate was precipitated as ammonium phospho-

molybdate, which was converted into magnesium pyrophosphate and weighed as such.

(6) *Total nitrogen.* This was determined by Kjeldahl's method, using 5 cc. of milk.

(7) *Ash.* A known weight of milk was ignited at a low temperature (Argand burner), and the ash determined by weighing until a constant weight was obtained.

(8) *Fat.* The fat was determined by Gerber's method.

Table I.

| Cow | Date | Quantity and kind of oil daily | Titratable acidity $N/9$ NaOH | Rennet test, minutes and seconds | Total calcium % | Diffusible calcium g. in 25 cc. | % total calcium diffused | Phosphorus % | Nitrogen % | Ash % | Fat % |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Flora I | 7. i. 27 | — | 0·140 | — | 0·129 | 0·00956 | — | 0·100 | — | 0·83 | 4·25 |
| Flora I | 18. i. 27 | 4 oz. C.L.O. | 0·155 | 4′ 28″ | 0·130 | 0·0109 | 32·37 | 0·103 | 0·49 | 0·82 | 3·70 |
| ,, | 1. ii. 27 | 6 ,, ,, | 0·120 | 5 5 | 0·137 | 0·0107 | 30·63 | 0·097 | 0·61 | 0·85 | 3·35 |
| ,, | 1. iii. 27 | 8 ,, ,, | 0·134 | 5 0 | 0·129 | 0·0106 | 32·18 | 0·090 | 0·61 | 0·79 | 3·55 |
| ,, | 30. iii. 27 | 8 ,, ,, | 0·123 | 7 15 | 0·126 | 0·0101 | — | 0·096 | 0·51 | 0·80 | 3·35 |
| ,, | 3. v. 27 | 8 ,, ,, | 0·100 | 12 30 | 0·128 | — | — | 0·118 | 0·60 | 0·86 | 4·50 |
| ,, | 6. vii. 27 | 4 ,, ,, | 0·129 | 7 35 | 0·123 | 0·0086 | — | 0·097 | 0·53 | 0·84 | — |
| Winsome II | 18. i. 27 | 4 ,, ,, | 0·174 | 2 20 | 0·127 | 0·0100 | 30·59 | 0·112 | 0·49 | 0·81 | 3·75 |
| ,, | 2. ii. 27 | 6 ,, ,, | 0·155 | 3 25 | 0·137 | 0·0094 | 26·58 | 0·099 | 0·64 | 0·86 | 3·65 |
| ,, | 2. iii. 27 | 6 ,, ,, | 0·134 | 5 5 | 0·144 | 0·0089 | 23·89 | 0·091 | 0·55 | 0·80 | 4·20 |
| ,, | 31. iii. 27 | 6 ,, ,, | 0·148 | 3 35 | 0·154 | 0·0093 | — | 0·125 | 0·54 | 0·79 | 4·10 |
| ,, | 3. v. 27 | 6 ,, ,, | 0·140 | 4 30 | 0·155 | — | — | 0·118 | 0·60 | 0·86 | 4·50 |
| ,, | 6. vii. 27 | 2 ,, ,, | 0·153 | 4 0 | 0·121 | 0·0088 | — | 0·104 | 0·54 | 0·80 | — |
| Flora II | 1. ii. 27 | None | 0·170 | 2 25 | 0·117 | 0·0109 | 35·97 | 0·100 | 0·63 | 0·78 | 4·50 |
| ,, | 1. iii. 27 | 4 oz. arachis | 0·168 | 2 30 | 0·119 | 0·0103 | 33·71 | 0·093 | 0·61 | 0·73 | 4·20 |
| ,, | 30. iii. 27 | None, but 8 oz. from 13 to 28. iii. 27 | 0·148 | 3 55 | 0·126 | 0·0104 | — | 0·113 | 0·56 | 0·78 | 5·15 |
| Clara | 2. ii. 27 | None | 0·175 | 2 20 | 0·116 | 0·0093 | 31·06 | 0·099 | 0·66 | 0·79 | 4·80 |
| ,, | 2. iii. 27 | 4 oz. arachis | 0·178 | 2 5 | 0·111 | 0·0088 | 30·83 | 0·098 | 0·55 | — | 4·80 |
| ,, | 31. iii. 27 | None, but 8 oz. from 13 to 30. iii. 27 | 0·158 | 2 30 | 0·123 | 0·0099 | — | 0·110 | 0·54 | 0·69 | 5·10 |

The results which were obtained are shown in Table I. In nearly every case except those of the rennet test and fat, the determinations were carried out in duplicate and the mean results only are given.

It is obvious that insufficient experiments have been made to justify any very definite statement of differences in the composition of some constituents of the milk, but certain results are somewhat striking and the general tendency of the effect of the feeding of the cod-liver oil in the quantities given may be summarised as follows.

(1) The acidity of the milk as determined by titration with $N/9$ NaOH is less when the cows are receiving cod-liver oil, although the differences are not greater than those which are sometimes found in comparing the milk of individual cows.

(2) The time of coagulation of the milk by rennet is increased very considerably.

(3) The total percentage of calcium is distinctly higher in the milk of the cows receiving cod-liver oil.

(4) Insufficient results were obtained to show definitely the relationship between the amount of the calcium which was diffusible and the total amount: moreover, as the specific gravity had not been determined experimentally, this was calculated from the fat and total solids, the amount of milk in the diffusion thimbles estimated from this theoretical value, and the figures given determined from these results. As far as can be judged from these inadequate data, it seems possible that the percentage of total calcium which is diffusible is less when cod-liver oil is fed than otherwise. If this were the case, it would help to explain the increase in the time of coagulation by rennet.

(5) The total phosphorus content appears to be unaffected.

(6) The total nitrogen content appears to be unaffected.

(7) The ash content is higher in the case of the cod-liver oil cows.

(8) The fat content is lower. This result is amply confirmed by the daily fat tests of the milk of individual cows.

An interesting variation which has been effected in the composition of the milk is the marked increase in the percentage calcium content.

The calculation of the total amount of calcium in the milk given on each occasion when an analysis was made showed that the increase in the percentage of calcium was not due to diminution in the yield of milk.

Since the foregoing work was carried out, the author has found that similar increases in the calcium content of the milk of lactating goats are shown in some of the results of Hart, Steenbock, Kletzein and Scott [1927]. The authors have not drawn attention to this point as they were investigating calcium assimilation and came to the conclusion that when the unsaponifiable fraction of cod-liver oil was fed in capsules it was not effective in improving calcium assimilation, but that when fed as a solution in corn oil it resulted in marked improvement in calcium assimilation.

Further, however, they stated that the corn oil itself had no effect, but if the figures which they quote are considered their results show that, in the case of Animal 1, the calcium in the milk increased from 0·128 to 0·133 % when a quantity of the unsaponifiable fraction corresponding to 40 cc. of cod-liver oil was fed daily in capsules (January 28–February 25); when an equivalent of 80 cc. was fed daily (February 25–March 18) this further increased to 0·146 % and when an equivalent of 80 cc. was fed in solution in 15 cc. of corn oil the percentage was still further raised to 0·148 % (March 18–April 29).

Similar results were obtained with Animal 2, whereas Animal 3 showed a diminution in calcium content of the milk under similar conditions.

With respect to the corn oil in which the cod-liver oil was fed, the authors state that it did not improve calcium assimilation, but from the single result given, it appears that it does cause an increase in the percentage calcium content of the milk, the milk of Animal 4 showing a percentage of 0·131 % when no corn oil was given (June 3 to July 1) which increased to 0·150 % when 15 cc. corn oil were fed (July 1 to 28).

It must be pointed out that these figures do not in any way contradict the conclusions of the authors (Hart, Steenbock, Kletzein and Scott) which are based on complete metabolism experiments, but seem to confirm our finding in that in two cases out of three the percentage of calcium in the milk of the lactating goats was considerably increased when cod-liver oil was added to their ration.

The results which have already been tabulated and discussed are based on analyses of evening milk; another series of calcium estimations was made on samples of morning milk of the same four cows and the results are shown in Table II.

Table II.

| Cow | Date | Additional ration | Titratable acidity | % total calcium |
|---|---|---|---|---|
| Flora I | 24. ii. 27 | 6 oz. cod-liver oil | 0·128 | 0·133 |
| ,, | 4. iii. 27 | 8 ,, ,, | — | 0·135 |
| ,, | 18. iii. 27 | 8 ,, ,, | 0·130 | 0·129 |
| Winsome II | 24. ii. 27 | 6 ,, ,, | 0·148 | 0·141 |
| ,, | 4. iii. 27 | 6 ,, ,, | — | 0·138 |
| ,, | 18. iii. 27 | 6 ,, ,, | 0·154 | 0·145 |
| Clara | 24. ii. 27 | 2 oz. arachis oil | 0·180 | 0·117 |
| ,, | 4. iii. 27 | 4 ,, ,, | — | 0·114 |
| ,, | 18. iii. 27 | 8 ,, ,, | 0·183 | 0·117 |
| Flora II | 24. ii. 27 | 2 ,, ,, | 0·155 | 0·118 |
| ,, | 4. iii. 27 | 4 ,, ,, | — | 0·121 |
| ,, | 18. iii. 27 | 8 ,, ,, | 0·171 | 0·120 |

It is seen that these results confirm those which have been already given, showing a distinctly higher percentage calcium content in the milk of the two cows which were receiving cod-liver oil in comparison with those two cows which were receiving arachis oil.

## Summary.

The analyses of the milk of cows which have been fed on well-balanced diets to which varying quantities of cod-liver oil and arachis (pea-nut) oil have been added, indicate that the addition of the cod-liver oil results in changes in the chemical composition of the milk, particularly an increase in the percentage of total calcium.

Insufficient results have as yet been obtained to justify any very definite conclusions, but in view of the fact that the butter made from the milk of cows fed on cod-liver oil is known to be much more strongly antirachitic than butter from the milk of cows which have not received cod-liver oil; the results already obtained seem to be of sufficient interest to warrant further analyses.

No suggestion is as yet put forward as to the source of the extra calcium which is found in the milk.

This paper is only of a preliminary nature and further work is now in progress.

In conclusion I wish to thank H. Hallett and A. Wagstaff for help in carrying out the necessary analyses.

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# XXII. DIGESTION IN THE COCKROACH.

## III. THE DIGESTION OF PROTEINS AND FATS.

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(*Received December 30th, 1927.*)

This paper concludes a comparative study of the digestive enzymes of the cockroach. In the first and second papers of the series [Wigglesworth, 1927, 1, 2] it has been shown that the crop contents of this insect are normally rendered acid ($p_H$ 4·6–6·0) by the action of micro-organisms upon the carbohydrates in the food, and that the enzymes concerned in carbohydrate digestion are active over this range of $p_H$, but, in other respects, resemble the corresponding enzymes from other sources. When the food consists of protein alone, the reaction of the crop is about 6·2. The present paper deals with the enzymes concerned in the digestion of proteins and fats.

### Proteolytic enzymes.

Preliminary qualitative tests gave results in agreement with those of Swingle [1925] and Abbott [1926], who state that the proteolytic enzymes of the cockroach are secreted only in the mid-gut and that pepsin-like enzymes are absent, digestion being most active in weakly alkaline solution. This is well seen in Table I which shows the digestion of fibrin at different $p_H$ values. Blood-fibrin was ground up into particles about the size of coarse sand, stained with Congo red by the method of Roaf [1909] and preserved in glycerol. For the experiment shown, the fibrin was washed, squeezed dry and weighed out in 0·075 g. quantities. These were mixed with 0·7 cc. of buffer solution (Clark and Lubs) and 0·1 cc. of a glycerol extract from the mid-gut of *Periplaneta americana* or 0·1 cc. of pancreatin in 50 % glycerol, and shaken in a water-bath at 37° for 24 hours. Control experiments with boiled enzyme preparations showed that "fibrolysis" [Long and Barton, 1914] played no part in the digestion observed.

Table I.

| | $p_H$ | 1·1 | 2·2 | 3·0 | 4·0 | 5·0 | 6·0 | 7·0 | 8·0 |
|---|---|---|---|---|---|---|---|---|---|
| Digestion after 24 hrs. | Cockroach protease | – | – | – | ± | + | ++ | +++ | ++ |
| | Pancreatic trypsin | – | – | – | – | ± | + | ++ | +++ |

At the end of this experiment the $p_H$ in the tubes at 2·2 and 3·0 was brought back to 7·0 by the addition of alkali. Twenty-four hours later there was well-marked digestion in both tubes. Like pancreatic trypsin the cockroach protease is inactivated, but is not destroyed, by moderate acidity. Like trypsin again the enzyme was found to be completely destroyed by keeping at about $p_H$ 12 at 37° for 30 minutes.

These results suggested that the protease of the cockroach is closely similar to mammalian trypsin; for although the former appeared to be rather more active in the acid direction, yet the maximal activity is well to the alkaline side of the isoelectric point of the protein substrate; and it is therefore clear that the enzyme does not resemble in this respect the plant proteases of the papain type [Willstätter and Grassmann, 1924]. A comparison of pancreatic trypsin and cockroach protease was therefore undertaken.

*Comparison of pancreatic trypsin and cockroach protease.*

(i) *Products of digestion of caseinogen.* A crude glycerol extract of cockroach mid-gut was incubated at 37° with 10 % caseinogen at about $p_H$ 7·5 with the addition of toluene. At the end of 10 days the mixture was heated on a boiling water-bath for an hour and filtered hot. The filtrate gave an intense bromine reaction for free tryptophan and after evaporating to small bulk and allowing to stand 24 hours, crystals of leucine and tyrosine separated out. These results are the same as those obtained with pancreatic trypsin.

(ii) *The relative production of "free acidity" and "formaldehyde acidity" during digestion.* Some years ago Mr S. W. Cole of the Biochemical Laboratory, Cambridge, observed an interesting distinction between peptic and tryptic digestion of proteins as followed by the formaldehyde titration of Sørensen. These observations were communicated to the Biochemical Society but have never been published, and I am indebted to Mr Cole for permission to reproduce here his original curves (Figs. 1 and 2).

When gelatin (or other protein) is acted upon by proteolytic enzymes, the quantity of standard alkali required to titrate the mixture to an arbitrary $p_H$ (say, 8·4) is found to increase as digestion proceeds. This may be provisionally termed "free acidity." If this mixture (at $p_H$ 8·4) be tested with neutral formaldehyde, the reaction becomes acid, due to the formation of methylene compounds, and a further amount of standard alkali is required to bring the mixture back to $p_H$ 8·4. This may be termed "formaldehyde acidity."

Figs. 1 and 2 show the "free acidity" and "formaldehyde acidity" produced during the digestion of gelatin by trypsin and pepsin respectively. It will be seen that in the case of trypsin, during the initial stages of digestion, the increase of the "free acidity" is rather more rapid than that of the "formaldehyde acidity," but that, whereas the former soon falls short and remains constant, the latter continues to increase. On the other hand, during

peptic digestion the "free acidity" far outstrips the "formaldehyde acidity." The same result is obtained in the digestion of albumin and caseinogen.

Although the explanation of this phenomenon is quite obscure (Cole inclines to the view that it indicates a chemical difference in the type of linkage which is broken by the two enzymes) yet it remains a very interesting empirical means of differentiating the two types of protein break-down.

Fig. 3 shows the digestion of albumin by the protease of the cockroach. It is clear that this is of the tryptic type.

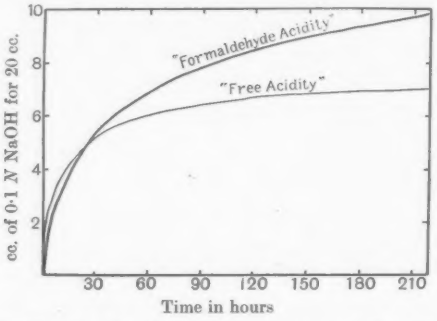


Fig. 1. Digestion of 4·5 % gelatin by trypsin at $p_H$ 8·1. (Cole.)

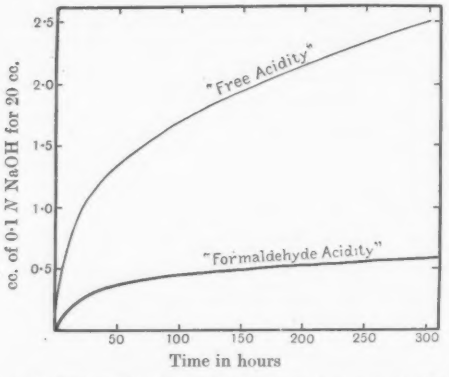


Fig. 2. Digestion of 4·5 % gelatin by pepsin at $p_H$ 1·4. (Cole.)

(iii) *Effect of salts.* While trypsin is not strikingly affected by the presence of salts, it is well known that papain is strongly activated by cyanides, and Ringer and Grutterink [1926] have shown that this enzyme is activated also by phosphates.

For the investigation of the effect of salts, the rate of clearing of a suspension of coagulated albumin has been used as a test of proteolytic activity. A 0·5 % solution of serum-albumin was placed in a boiling water-bath and

traces of dilute hydrochloric acid added very cautiously until a deep cloud, without any flocculation, was produced. 0·5 cc. quantities of this suspension were measured into uniform small test-tubes (100 by 6 mm.) and the volume made up to 1·0 cc. with the enzyme and other reagents under investigation. A standard tube was prepared by taking 0·25 cc. of the albumin suspension and making up to 1·0 cc. with distilled water. The time required for the degree of clearing in the experimental tubes to equal that of the standard, gave a measure of the enzyme activity. The opacity of the two tubes could be compared very accurately by looking through each at two identical words printed in small type on white paper.

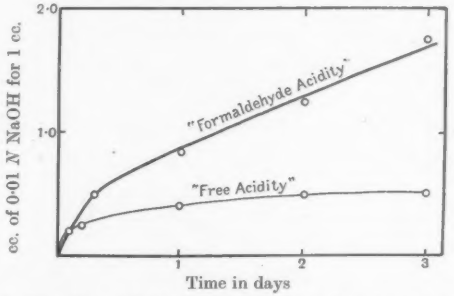


Fig. 3. Digestion of 3 % albumin by cockroach protease at $p_H$ 7·6.

By this means the activity of cockroach protease at $p_H$ 6·0 in the presence of phosphate and phthalate buffers (about 0·04 *M*) was compared and found to be identical; similarly at $p_H$ 8·0 in the presence of phosphate and borate buffer.

Hydrocyanic acid had no activating effect but, as in the case of trypsin, caused some inhibition. This is shown in Table II.

Table II.

| Concentration of KCN g. per 100 cc. | Relative velocity at $p_H$ 7·0 | |
|---|---|---|
| | Trypsin | Cockroach protease |
| 0 | 100 | 100 |
| 0·2 | 87 | 86 |
| 0·5 | 70 | 60 |

(iv) *The $p_H$-activity curves with different substrates.* The preliminary experiments with coloured fibrin, described above, seemed to indicate that the range of activity of cockroach protease extends further in the acid direction than that of pancreatic trypsin. Experiments made to investigate this point more carefully are given in Figs. 4, 5, 6 and 7.

Fig. 4 shows the digestion of serum-albumin. This was carried out by the opacity method described in the last section, after it had been shown that the initial opacity was not affected by the range of $p_H$ used and that flocculation did not occur until the $p_H$ was below 6·0.

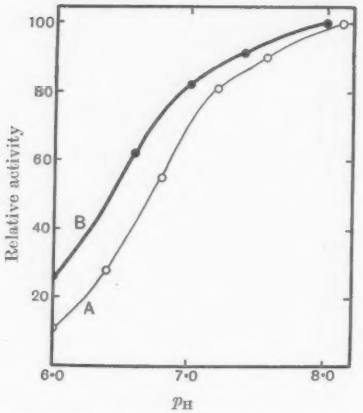


Fig. 4. $p_H$-activity curves of digestion of serum-albumin (0·5 %). A, Trypsin; B, Cockroach protease.

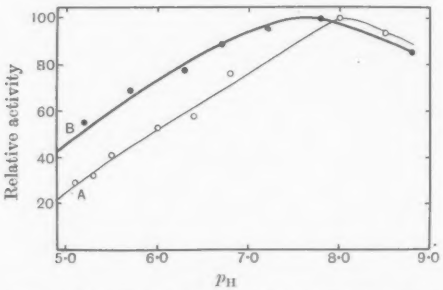


Fig. 5. $p_H$-activity curves of digestion of gelatin (5 %). A, Trypsin; B, Cockroach protease.

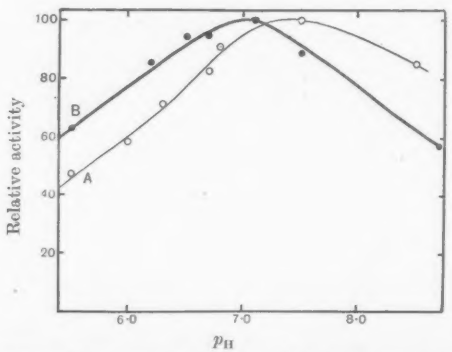


Fig. 6. $p_H$-activity curves of digestion of caseinogen (5 %). A, Trypsin; B, Cockroach protease.

For the observations on gelatin, caseinogen and edestin (Figs. 5, 6 and 7) the formaldehyde titration was used. The experimental mixtures consisted of 0·1 cc. of enzyme, 0·4 cc. of an appropriate buffer and 0·5 cc. of substrate. After the addition of toluene the mixtures were shaken in a water-bath at 37°. At the start and at the end of 24 hours, 0·1 cc. of each mixture was measured into 5 cc. of distilled water and the formaldehyde titration carried out with $N/100$ NaOH. The $p_H$ was determined colorimetrically after diluting with 8 volumes of distilled water. During the course of the experiment there was an increase in acidity which varied between $p_H$ 0·1 and 0·3. The figure used in the construction of the curves is the mean between those obtained at the beginning and at the end of the experiment.

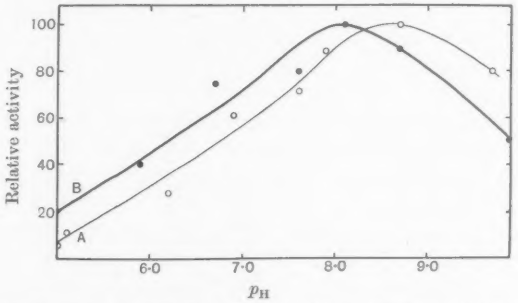


Fig. 7. $p_H$-activity curves of digestion of edestin (2 %). A, Trypsin; B, Cockroach protease.

The experiments show consistently that with a given protein the range of activity of the cockroach protease extends further in the acid direction than is the case with pancreatic trypsin.

This result is of considerable interest; for Northrop [1922] maintains that the form and position of the $p_H$-activity curve of trypsin is determined solely by the titration curve of the substrate protein. Were this the case, it would follow that, under the same experimental conditions, the $p_H$-activity curves of trypsins from different sources should be identical. The above experiments, however, show that the trypsin of the cockroach is more active in the acid range than is pancreatic trypsin and they suggest that, as is the case with the enzymes acting upon carbohydrates, the $p_H$-activity curve is determined in part at least by the nature of the enzyme or, as is the case with lipolytic enzymes, by the nature of associated substances. On the other hand the results are in general agreement with those of Northrop, for it will be seen that it is proteins such as gelatin and caseinogen, whose dissociation curves are further in the acid zone, which are the more readily digested in acid solution; while edestin with a less acid isoelectric point is less readily digested at the lower $p_H$.

*Erepsin.*

Swingle [1925], on the grounds that an extract from the gut of the cockroach would cause an increase in the formaldehyde titration of a solution of peptone, and would produce, in this digest, nitrogenous compounds not precipitated by phosphotungstic acid, states that an erepsin is present in the alimentary tract of this insect. The work of Waldschmidt-Leitz and Harteneck [1925] has resulted in a more precise definition of erepsin (peptidase) as an enzyme capable of hydrolysing only dipeptides and tripeptides, substances upon which trypsin is without any action. There is in fact no overlap in the range of action of the two enzymes. It seemed desirable therefore to investigate again the peptidase of the cockroach.

For qualitative experiments, as advocated by Pfeiffer and Standenath [1925], the dipeptide, glycyltryptophan, has been employed. A solution of this dipeptide ("Fermentdiagnostikum") was kindly supplied by Messrs Bayer Products Ltd.

The mid-gut of a single *P. americana* was ground up in 1·0 cc. of water with a little thymol. 0·1 cc. of this emulsion was mixed with 0·4 cc. of water and 0·1 cc. of glycyltryptophan and incubated at 37°. A similar mixture containing boiled emulsion was used as a control. At intervals, 0·1 cc. was withdrawn, mixed with a drop of 5 % acetic acid, and minute traces of bromine water added. The test mixture showed a faint pink at the end of 2 hours and an intense pink after 24 hours. The control showed no change. It is clear that the gut of the cockroach contains a true erepsin. Further experiments conducted in the same manner showed that, as was shown by Swingle [1925] to be the case with the other digestive enzymes, the erepsin is actually secreted into the gut contents.

An attempt was made to separate this enzyme from the trypsin above described. For this purpose the technique of Waldschmidt-Leitz and Harteneck [1925] was followed. Sixteen cockroaches (*P. americana*) were dissected and the mid-guts ground up with thymol and glycerol, made up to 16 cc. with 50 % glycerol and filtered through cotton wool. This extract gave positive tests for trypsin and for peptidase. 8 cc. of this extract, with 0·5 cc. of phthalate buffer, $p_H$ 4·6, were shaken up with 1·5 cc. of freshly prepared aluminium hydroxide[1], allowed to stand 2 minutes and centrifuged. This procedure was repeated twice upon the supernatant fluid which was then brought to about $p_H$ 7·0 and will be referred to as preparation A. The precipitate of aluminium hydroxide in the original mixture was extracted for 2 hours with 8 cc. of $N/25$ ammonia in 18 % glycerol. The mixture was then centrifuged and the supernatant fluid brought to about $p_H$ 7·0 (preparation B).

Tables III and IV show the results of tests applied to the resulting preparations.

[1] 6·8 g. of aluminium sulphate were dissolved in 40 cc. of water and $N/5$ ammonia added to 100 cc.

Table III. *Mixtures:* 0·1 *cc. of enzyme preparation* + 1·0 *cc. of calcified milk diluted* 1 *in* 10.

| | Preparation A | | Preparation B | |
|---|---|---|---|---|
| | Test mixture | Boiled control | Test mixture | Boiled control |
| 9.15 a.m. | — | — | — | — |
| 9.20 „ | Clotting complete | — | — | — |
| 10.45 „ | „ „ | — | Clotting beginning | — |
| 11.15 „ | „ „ | — | „ complete | — |

Table IV. *Mixtures:* 0·1 *cc. of enzyme preparation* + 0·4 *cc. of phosphate buffer* $p_H$ 6·6 + 0·1 *cc. glycyltryptophan.*

| | Preparation A | | Preparation B | |
|---|---|---|---|---|
| | Test mixture | Boiled control | Test mixture | Boiled control |
| 9.45 a.m. | — | — | — | — |
| 6.0 p.m. | — | — | Faint pink | — |

It is clear from these experiments that a partial separation of the enzymes has been accomplished. Preparation A containing most of the trypsin and B containing most of the peptidase; *i.e.* in their adsorption by aluminium hydroxide they behave like the trypsin and erepsin of the pancreas as studied by Waldschmidt-Leitz and Harteneck.

Few comparative studies on peptidases have been made. Dernby [1917] compared yeast erepsin and gut erepsin and showed that they have the same $p_H$ optimum (7·8) but differ in the effect of neutral salts. Yeast erepsin is unaffected by a salt concentration up to 0·5 *M* while gut erepsin is markedly depressed by 0·02 *M* salt concentration.

For the investigation of the $p_H$ range of the cockroach erepsin, glycylglycine has been used as substrate and the digestion followed by formaldehyde titration. As described by Dernby the solution becomes more alkaline as digestion proceeds. Under the conditions of experiment this caused an increase in $p_H$ of 0·5 in the mixture showing the greatest change. The figures used in the construction of the curve (Fig. 8) are the means of the $p_H$ values, determined colorimetrically, at the beginning and at the end of the experiment 24 hours later. For the experiment given in Fig. 8 the mid-gut of a single *P. americana* was ground up in 1 cc. of water with thymol. The mixtures consisted of 0·1 cc. of this enzyme solution, 0·3 cc. of buffer and 0·4 cc. of 2 % glycylglycine. Owing to shortage of material this experiment was the only one performed. It shows an optimal zone around $p_H$ 8·5.

### Lipase.

If the cockroach be kept without food after a meal of carbohydrates, the crop is usually empty again at the end of 48 hours; but this is not the case after the ingestion of fat. Sandford [1918] states that a large meal of fat may remain in the crop of *P. americana* for 2 months, though Abbott [1926] working with *P. australasiae* failed to observe its persistence for such

long periods. These observations suggest that either the cockroach gut is poor in lipase, or the enzyme is acting in an unsuitable medium.

An active lipase was discovered by Plateau [1873] in extracts from the gut of the cockroach, and since that time there has been a considerable controversy as to the distribution of lipase and the seat of absorption of fat. The subject is reviewed by Abbott [1926]. Sandford [1918] and Swingle [1925] assert that the crop is of equal importance to the mid-gut in the secretion of lipase; but Abbott [1926], using *P. australasiae*, has shown that, as is the case with the enzymes acting upon carbohydrates, crop lipase is due entirely to regurgitation from the mid-gut.

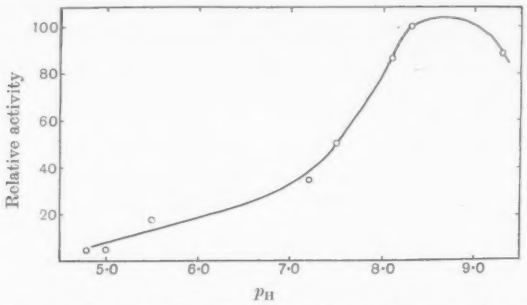


Fig. 8. $p_H$-activity curve of digestion of glycylglycine by cockroach erepsin.

Table V shows how the relative lipolytic activities of the crop and mid-gut of *Blatella germanica* are affected when the crop is washed out by giving water but no food for a fortnight. In each case the crops and mid-guts of a number of insects were separated, made up to an equal volume with water, and the lipolytic activity of the two resulting extracts compared as described below.

Table V.

| Notes | Relative activity: Crop | Relative activity: Mid-gut |
|---|---|---|
| Freshly caught *B. germanica* | 67 | 100 |
| Same batch after having water alone for 15 days | 18 | 100 |

These results support those of Abbott; but it is clear that lipase is removed from the crop with more difficulty than are the carbohydrate enzymes; and the possibility remains that some of the residual lipase demonstrated in Table V is due to intracellular enzyme in the cells of the crop, which would not be discharged into the gut.

Abbott has also shown conclusively that whereas the chitinous lining of the crop renders it quite impermeable to sugars and other substances in aqueous solution, it is readily permeable *in vitro* to fats and fat solvents, and that in the living animal, undigested fat passes through the chitinous lining

and is taken up by the cubical cells of the crop wall. Any deficiencies in the digestion of fat may therefore perhaps be compensated to some extent by the absorption of undigested material.

The comparative study of lipases is rendered difficult and unsatisfactory by the great extent to which their properties are affected by associated substances, so that a lipase from a given source may vary greatly in its properties depending upon its degree of purification. This applies to the optimum range of $p_H$ [Willstätter, Haurowitz and Memmen, 1924], to the effect of bile salts [Willstätter and Memmen, 1924] and to the relative activity upon lower esters and the glycerides of the higher fatty acids [Platt and Dawson, 1925].

No great significance therefore can be attached to the experiments given here, where the lipase in a crude aqueous emulsion of cockroach mid-gut has been compared with an aqueous solution of pancreatin.

*Methods.* Enzyme preparations from the cockroach consisted of the mid-gut of a single *P. americana* freshly ground up with a little thymol in 1 cc. of water to give a uniform emulsion. Pancreatic lipase was obtained by dissolving pancreatin in water and neutralising to about $p_H$ 7·0.

Lipolytic activity has been measured by the production of acid from ethyl butyrate. 0·2 cc. of enzyme was mixed with 0·8 cc. of buffer solution (Clark and Lubs) in a small test-tube, and 0·1 cc. of neutralised ethyl butyrate added. 0·1 cc. of this mixture was withdrawn, added to 5 cc. of distilled water and titrated with $N/100$ NaOH to $p_H$ 9·0 using thymol blue as indicator. The mixtures for comparison were shaken side by side in a water-bath at 37° and the titration repeated at the end of one or two hours. Under these conditions the change in $p_H$ during the experiment seldom exceeded 0·4. In a few experiments a 25 % emulsion of olive oil was used in place of the ethyl butyrate.

(i) *Effect of bile salts.* There is considerable uncertainty as to the effect of bile salts upon lipase. The digestion of true fats is said to be increased by the improved emulsification in the presence of bile salts; and in addition, bile salts are believed by Platt and Dawson to have a specific chemical effect in stimulating lipase, as evidenced in the hydrolysis of ethyl butyrate. Abbott observed a definite increase in the breakdown of olive oil by the lipase of the cockroach in the presence of ox bile.

The results obtained with sodium glycocholate (0·1 %) and fresh aqueous extracts of cockroach mid-gut have been variable and often uncertain; but usually there has been increased action upon both olive oil and ethyl butyrate. The same variability occurs in experiments with pancreatic lipase.

(ii) *Effect of phosphates.* Platt and Dawson hold that phosphates have a specific activating effect upon pancreatic lipase, independent of their influence as buffers.

This observation has been confirmed in the case of pancreatic lipase but could not be demonstrated with the crude cockroach lipase. The mixtures

used are given in Table VI. At the end of the experiment the $p_H$ in the phosphate and phthalate mixtures was shown to be the same.

Table VI. 0·1 *cc. enzyme* + 0·9 *cc. buffer*, $p_H$ 6·0, + 0·1 *cc. neutralised ethyl butyrate.*

| | | Increase in acidity at end of 2 hrs. $N/100$ NaOH for 1 cc. | |
|---|---|---|---|
| Pancreatic lipase. | Phosphate buffer | (*a*) 1·8 | (*b*) 1·7 |
| ,, | Phthalate ,, | (*a*) 1·3 | (*b*) 1·3 |
| Cockroach lipase. | Phosphate ,, | (*a*) 1·0 | (*b*) 1·1 |
| ,, | Phthalate ,, | (*a*) 1·1 | (*b*) 1·1 |

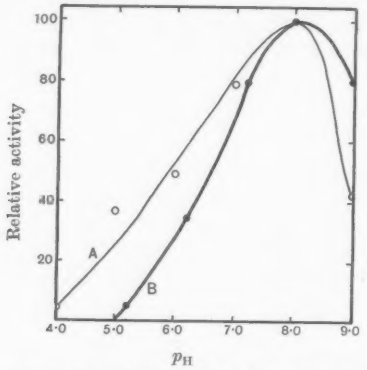


Fig. 9. $p_H$-activity curve of hydrolysis of ethyl butyrate by pancreatic lipase (A) and cockroach lipase (B).

(iii) $p_H$-*activity curve.* Fig. 9 shows the $p_H$-activity curve for pancreatic lipase compared with that for the crude extract of cockroach gut. The curve (A) for pancreatic lipase agrees fairly well with that obtained by Platt and Dawson for a crude glycerol extract of pig's pancreas. But for the reasons stated in the introductory paragraph on lipase, the precise position of these curves is of little significance. One fact, however, is clear; the lipase of the cockroach is completely inactive at $p_H$ 5·0 and is therefore singularly ill-adapted to function in the crop of the living insect where the $p_H$ 24 hours after a meal of fat varied in several insects from 4·8 to 5·2.

## Summary.

1. The proteolytic enzymes of the cockroach consist of a tryptase and a peptidase. Peptase is absent.

2. The tryptase resembles pancreatic trypsin in (*a*) the products of digestion, (*b*) the relative production of "free acidity" and "formaldehyde acidity" during digestion, (*c*) the effect of salts and (*d*) in acting upon proteins only on the alkaline side of their isoelectric point.

3. The two enzymes differ in that with a given protein the range of activity of the cockroach protease extends further in the acid direction. The theoretical significance of this fact is discussed.

4. The peptidase is present in the intestinal secretion. It has been partially separated from the tryptase by adsorption. From a single experiment it appears to have an optimal zone of activity about $p_H$ 8·5.

5. Lipase, as present in a crude emulsion of the cockroach gut, is usually, but not constantly, activated by sodium glycocholate; it is not activated by phosphates apart from their influence as buffers, and it shows an optimal $p_H$ zone around 8·0. It is almost inactive at the normal reaction of the crop.

The author is indebted to Mr H. R. Catchpole of Downing College and Mr G. D. Emms of St John's College for a supply of glycylglycine.

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# XXIII. THE DETERMINATION OF AMINO-ACIDS IN THE BLOOD: A NOTE ON FOLIN'S METHOD.

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(*Received December 30th, 1927.*)

Two methods of estimating the amino-acid content of blood are in use at the present time: that of Van Slyke and Meyer [1912], gasometrically, and that of Folin [1922], colorimetrically. Of these the latter, on account of its simple technique, is the more familiar, and since its introduction has been used by the majority of workers.

The investigations reported in this paper arose from the observation that all the amino-acid figures obtained by us in the course of a research on certain diseases of children were higher than those quoted by Folin for young men. This applied not only to the pathological cases under examination, but to a series of controls taken from normal school-children, and to some normal adult bloods used as further controls.

In a review of the somewhat scanty literature of the subject to ascertain a definite normal we found that although the majority of workers seemed to obtain figures roughly between 6 and 7 mg. amino-acid-N per 100 cc. of blood, there were sufficient exceptions to justify an examination of any sources of error which the method might possess. Appended are some results taken from papers by Hoeffel and Moriarty [1924], Greene, Sandiford and Ross [1924], Swanson [1924], Lennox [1926], Becher and Herrmann [1925], Plass and Matthew [1925], and Folin and Berglund [1922].

Table I.

| Investigators | No. of estimations | Amino-acid-N mg. % | | |
|---|---|---|---|---|
| | | Max. | Min. | Mean |
| Swanson | 19 | 11·0 | 5·5 | 7·47 |
| Hoeffel and Moriarty | 9 | 8·12 | 6·38 | 7·04 |
| Folin and Berglund | 12 | 7·8 | 5·7 | 6·4 |
| Greene, Sandiford and Ross | 20 | 7·2 | 5·2 | 6·37 |
| Lennox | — | 7·3 | 5·1 | — |
| Becher and Herrmann | — | 6·7 | 6·0 | — |
| Plass and Matthews | — | 7·0 | 5·3 | 6·1 |

Our own results may be classified in five groups, as in Table II.

Table II.

| Type of blood | | No. of estimations | Amino-acid-N mg. % (mean) |
|---|---|---|---|
| *Children:* | | | |
| Pathol. | Fasting | 77 | 8·23 |
| Do. | Non-fasting | 38 | 9·02 |
| Normal. | Fasting | 17 | 8·32 |
| *Adults:* | | | |
| Normal. | Fasting | 7 | 8·62 |
| Do. | Non-fasting | 5 | 9·41 |

It was in the hope of finding an explanation not only of our own high results but of the differences between those of other workers that we undertook the research here described.

Details of the method will not be given here, as it will be found fully described in most text-books of practical biological chemistry. It is dependent upon the production of a brightly coloured compound by the action of a quinone derivative upon the amino-group in alkaline solution. Instructions are given by Folin for producing the necessary alkalinity, as follows. The standard solution is made up in $N/10$ HCl, and 1 cc. taken in the experiment. To it is added 1 cc. of sodium carbonate of a definite strength. The blood filtrate is brought to the same degree of alkalinity by addition of a few drops of the same carbonate solution, a drop of phenolphthalein in each solution serving as indicator.

It was upon this part of the estimation that we settled as being in all probability the source of anomalous results, through an observation made during our experiments on adult controls. The samples were taken mainly from members of the hospital staff. In the course of these estimations a fresh solution of sodium carbonate was prepared, rather weaker than that used in the previous work. It was noticed that the amino-acid results fell simultaneously, both for subsequent normals and for pathological specimens. Table III gives the results of our normal adult controls.

Table III.

| Blood sample | Sex | | Amino-acid-N mg. % |
|---|---|---|---|
| S. H. E. 1 | M. | Non-fasting | 9·69 |
| S. H. E. 2 | | ,, | 9·27 |
| V. S. M. | M. | ,, | 9·52 |
| O. G. C. | F. | ,, | 9·79 |
| H. G. P. | M. | ,, | 8·79 |
| A. G. O. | M. | Fasting | 8·86 |
| H. P. J. | M. | ,, | 9·52 |
| *J. E. G. | F. | ,, | 7·29 |
| *D. G. H. | F. | ,, | 6·22 |

* Less alkaline solution.

The drop of 2 to 3 mg. observed in the last two bloods was so marked, being outside the limit of experimental error, that an estimation was carried out on two portions of another filtrate at different alkalinities.

Result: for less alkaline solution, 7·11 mg.; for more alkaline, 8·80 mg.

To establish this definitely five normal bloods were taken from men students of the medical school. These were all fasting specimens drawn before breakfast. Two standards were prepared, containing 1 cc. of the stronger carbonate and 1 cc. of the weaker respectively. Two portions of each filtrate were taken, one being matched against each standard. Results are given in Table IV.

Table IV.

| Sample | Amino-acid-N mg. % | | |
|---|---|---|---|
| | Weaker solution | Stronger solution | Difference |
| B——n | 7·57 | 8·69 | 1·12 |
| D——l | 7·57 | 8·72 | 1·15 |
| H——l | 6·96 | 8·05 | 1·09 |
| K——n | 7·33 | 8·48 | 1·15 |
| M——t | 7·07 | 8·00 | 0·93 |

Not only does the more alkaline solution yield a higher amino-acid figure, but the increase is constant within experimental error for four of the five samples.

It next appeared desirable to find the variation of amino-nitrogen with alkalinity. To do this a normal adult blood was used and five portions taken. Five standards were set up, and to them added in order 1, 1·05, 1·10, 1·15 and 1·20 cc. of sodium carbonate. One portion of filtrate was matched against each standard, and on the completion of the experiment each was read against its own standard. The alkali was added from a micro-burette.

Table V gives the results obtained.

Table V.

| Cc. alkali added | Amino-acid-N mg. % estimated |
|---|---|
| 1·00 | 6·66 |
| 1·05 | 7·18 |
| 1·10 | 7·77 |
| 1·15 | 8·00 |
| 1·20 | 8·14 |

It will be observed that the extra amount of amino-nitrogen estimated for each increment of carbonate becomes less with increasing alkalinity. We should expect therefore that at some higher alkalinity a maximum would be obtained. To prove this an experiment of larger range was carried out, using the same technique.

Table VI.

| Cc. alkali added | Amino-acid-N mg. % | |
|---|---|---|
| | Estimated | Increase |
| 1·00 | 6·68 | — |
| 1·05 | 7·00 | 0·32 |
| 1·10 | 7·95 | 0·95 |
| 1·15 | 8·14 | 0·19 |
| 1·30 | 8·64 | 0·50 |
| 1·50 | 8·86 | 0·22 |
| 1·80 | 8·97 | 0·11 |
| 2·00 | 8·92 | −0·05 |

If this is expressed in the form of a graph, plotting the volume of carbonate added against the amount of amino-acid-N, a curve is obtained, shown in Fig. 1. A smooth curve drawn through the points as in the figure is asymptotic to a line corresponding to a value for the amino-acid-N of between 8·90 and 8·95 mg. %. This smooth curve may be used to find intermediate points, and to correct those values already given. If we do this we obtain Table VII.

Table VII.

| Cc. alkali added | Amino-acid-N mg. % | |
|---|---|---|
| | From graph | Increase |
| 1·0 | 6·68 | — |
| 1·1 | 7·80 | 1·12 |
| 1·2 | 8·36 | 0·56 |
| 1·3 | 8·64 | 0·28 |
| 1·4 | 8·78 | 0·14 |
| 1·5 | 8·86 | 0·08 |
| 1·6 | 8·90 | 0·04 |
| 1·7 | 8·92 | 0·02 |

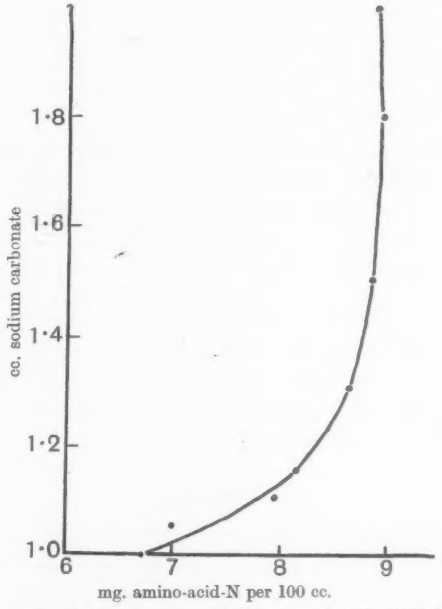


Fig. 1. Variation of amino-acid-N estimated with alkalinity.

Repetitions of this experiment yielded curves of similar type, although we did not succeed in obtaining another series as easy of mathematical interpretation as that in Table VII. Experimental error in the second place of decimals prevents much reliance being placed on the last three terms of

column 3, but allowing for this it will be seen that the increases form a geometrical progression for the first four terms, of ratio 1/2. The amino-acid-N series is therefore a progression described by the formula

$$u_n = ar^{n-1} + b$$

where $r$ is the ratio, 1/2 in this series, and $a$ and $b$ are constants, $u_n$ being the value of the $n$th term in the series. By substituting actual values for $n$ from the table $a$ and $b$ are found, and the amino-acid-N at infinite alkalinity is shown to be 8·92 mg. %. This, of course, is the asymptote to the curve.

Folin in his original paper gives exact details for obtaining the desired alkalinity. After giving directions for checking the strength of the sodium carbonate solution by titration, he says, "The correct degree of alkalinity is obtained when 1 cc. of this sodium carbonate solution is added to 1 cc. of amino-acid solution which is at the same time a 0·1 *N* solution of hydrochloric acid."

We have shown in the foregoing results that a variation from this alkalinity produces different readings in the amino-acid-N. In the paper from which we have quoted it is not stated upon what evidence the alkalinity there described is assumed to be the correct one; that is, no experimental proof is there given to show why this particular concentration of sodium carbonate had been chosen. It is in this respect, we feel, that the description published by Folin requires explanation.

It is clear that a series of estimations carried out with the same solutions of standard and sodium carbonate will yield results perfectly satisfactory for comparison one with another, even if one or other of the solutions should vary slightly from the concentration prescribed by Folin.

The fact that increase of alkalinity favours the speed at which the colour develops might be considered to explain our readings. To decide this point we performed an experiment similar to that in Table VI, allowing the solutions 40 hours in which to attain equilibrium. The results were again similar to those found after normal periods of development. Hence the time factor cannot be considered to have caused the variations in amino-acid-N with alkalinity which we have described.

## Summary.

Estimations of the amino-acid-N in blood filtrates have been carried out by Folin's method. It has been found that the figures obtained vary according to the alkalinity of the solutions, and that, *ceteris paribus*, an increase in the amino-acid-N determined follows from increasing the alkalinity.

The relative rates of change have been investigated, and it has been shown that the rate of increase of the amino-acid-N falls off gradually, to give a constant value at an alkalinity higher than that used by Folin.

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# XXIV. THE AMINO-ACID CONTENT OF THE BLOOD OF CHILDREN IN HEALTH AND IN DISEASE.

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(*Received December 30th, 1927.*)

The majority of the results in the following paper were obtained in the course of a research, still in progress, on the blood picture of rheumatism in childhood. Together with these, estimations were carried out upon other hospital cases of various kinds, and a series of controls upon normal children was later made.

The only previous work on this subject which we have found is given in a paper by Hoeffel and Moriarty [1924] giving the amino-acid-nitrogen content of the blood for normal infants and children. The figures there quoted are, for infants up to 22 months, 5·57 mg. per 100 cc.; for children of 2 years to 15 years, 5·28, and for adults, 7·04.

In our work blood samples were drawn, from the basilic vein, into oxalated tubes, and a tungstic acid filtrate prepared by the Folin-Wu method. The estimations were carried out by Folin's volumetric method [1922] as were those of Hoeffel and Moriarty.

## Effect of fasting.

The question of the possible increase of the amino-acid-nitrogen during protein ingestion has been debated by various workers. Hammett [1920] definitely denies that any difference can be found between blood specimens taken fasting and 3 hours after a meal. Folin and Berglund [1922], on the contrary, obtain a definite increase after a meal, and are supported by several other investigators. For some time our samples were taken at any convenient hour, and were therefore for the most part non-fasting. It was then decided to take all future specimens definitely fasting, *i.e.* before breakfast.

It must be remembered in studying the figures given below, that the amino-acids disappear rapidly from the blood, owing firstly to deaminisation by the liver, and secondly to absorption by the muscles. Our readings show that in spite of this, the blood figures are altered by a meal.

A number of results are given in Table I fasting and non-fasting. Though the limited number of figures for which there is here space is insufficient to

yield a fair average, a mean of the total estimations carried out in the research is given for each set of figures.

Table I.

| Specimen | Age | Sex | Diagnosis | Amino-acid-N mg. % |
|---|---|---|---|---|
| *Fasting:* | | | | |
| C. A. | 11 | F. | Chorea | 7·37 |
| B. L. | 5 | F. | ,, | 9·09 |
| V. W. | 12 | M. | ,, | 8·48 |
| N. P. | 10 | F. | Rheum. carditis | 7·63 |
| R. B. | 5½ | M. | ,, | 8·08 |
| J. M. | 12 | F. | Ac. rheum. + carditis | 8·46 |
| H. M. | 9 | M. | ,, | 9·21 |
| E. F. | 3 | F. | Pyelitis | 9·00 |
| W. H. | 5 | M. | Ac. nephritis | 7·70 |
| R. L. | 9 | M. | ,, | 6·55 |
| L. B. | 10 | F. | Renal rickets | 8·00 |
| C. Y. | 10 | F. | ,, | 6·25 |
| B. O. | 12 | F. | Coeliac rickets | 6·09 |
| L. D. | 10 | M. | Jaundice | 9·66 |
| G. T. | 12 | F. | Diabetes | 7·70 |
| *Non-fasting:* | | | | |
| J. L. | 7 | F. | Chorea | 10·00 |
| M. M. | 5½ | F. | ,, | 10·66 |
| F. D. | 8 | F. | ,, | 6·74 |
| V. G. | 11 | M. | Rheum. carditis | 9·60 |
| F. T. | 12 | M. | ,, | 10·40 |
| A. T. | 7 | F. | ,, | 9·67 |
| L. W. | 11 | F. | Chr. pyelitis | 10·17 |
| G. J. | 11 | F. | Rickets | 9·48 |
| T. S. | 4 | M. | Tetany | 8·70 |
| J. P. | 10 | M. | Chr. nephritis | 7·35 |
| K. S. | 4 | F. | Ac. nephritis | 7·73 |
| I. H. | 12 | F. | Exophthalmic goitre | 11·50 |
| E. D. | 11 | F. | Acholuric jaundice | 10·80 |
| J. B. | 7 | F. | Coeliac rickets | 8·07 |
| N. P. | 6 | F. | Renal rickets | 9·03 |
| Mean of 40 non-fasting bloods ... ... ... ... ... | | | | 8·97 |
| Mean of 77 fasting bloods ... ... ... ... ... ... | | | | 8·23 |

Where a considerable variation exists, as in the above series of results, it is more illuminating to record the proportion in which the readings are distributed in the scale of results. This is done in Figs. 1 and 2 which explain themselves. The number of results lying between each pair of limits 7 to 8, 8 to 9, and 9 to 10 mg. and the total number above and below the extreme limits, are represented, as percentages of the total obtained, by the height of the columns.

It is evident at once that the effect of protein digestion is to raise the amino-acid-nitrogen. In the non-fasting bloods the greatest number of results lie above 10 mg. (mainly between 10 and 11), the next greatest between 8 and 9, and the least below 7. For the fasting bloods, the greatest number lie between 8 and 9, the next greatest between 7 and 8, and the least above 10.

Nearly 48 % of the non-fasting bloods, but only 29 % of the fasting bloods, lie above 10 mg.

Conclusions drawn from a large range of results such as these seem to us of more value than isolated experiments involving the estimation of amino-nitrogen before and after protein feeding to selected cases, the method on which previous statements have mainly been based.

In order to obtain a conception of the difference in results attributable to digestion, it is preferable to take a less diversified series of cases. The ideal desideratum, namely, two series of normals, fasting and non-fasting, was unobtainable. For our purpose we have taken two series of results from cases of rheumatism and chorea, in which, as we shall show later, the amino-acid-nitrogen is normal.

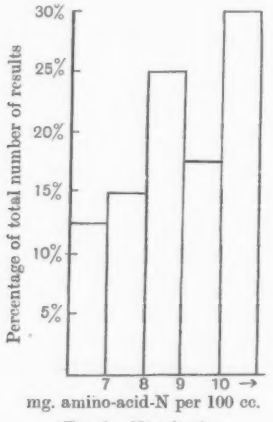


Fig. 1. Non-fasting.

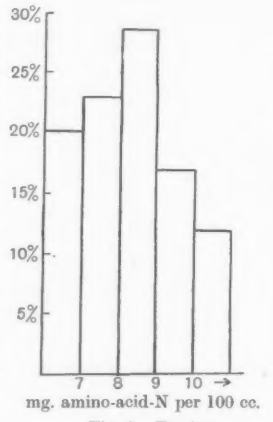


Fig. 2. Fasting.

The fasting figures lie between 6·75 mg. and 10·0, the non-fasting between 6·85 and 10·65. Of the fasting specimens 38·8 % lie above and 61·1 % below 8·5, while of the non-fasting specimens, 61·1 % lie above and 38·8 % below.

To procure reliable controls for the examination of these results, it was decided to utilise perfectly healthy children in preference to convalescent or "surgical" hospital cases.

Volunteers were obtained from school children of both sexes, attending at an elementary school in the neighbourhood, by the assistance of Dr G. A. Auden, school medical officer for Birmingham, to whom the author wishes to express his considerable indebtedness for the help given and interest taken in this matter.

The children attended at the hospital before breakfast and a blood sample was taken in the usual manner. Table II shows the results obtained.

It will be seen that these results are all higher than those given by Hoeffel and Moriarty, and, indeed, higher than any adult amino-acid-nitrogen figures given in the literature. This matter was investigated, and led to a separate research into the principles of the method employed, the results of which

Table II. *Normal children (fasting).*

| | Age | Sex | Amino-acid-N mg. % |
|---|---|---|---|
| M. Bh. | 11 | F. | 9·52 |
| E. Ws. | 10 | F. | 9·21 |
| J. B. | 13 | M. | 8·97 |
| A. N. | 11½ | M. | 8·91 |
| J. M. | 11 | M. | 8·86 |
| P. H. | 10 | F. | 8·70 |
| J. T. | 10 | M. | 8·58 |
| A. C. | 11 | M. | 8·43 |
| E. M. | 11½ | M. | 8·33 |
| F. R. | 11 | M. | 8·09 |
| P. P. | 10 | M. | 8·09 |
| E. Wd. | 11 | F. | 8·09 |
| M. Bs. | 11 | F. | 8·00 |
| F. C. | 11 | M. | 7·65 |
| P. E. | 11 | M. | 7·60 |
| E. B. | 10 | F. | 6·08 |
| Mean (16 estimations) | ... | ... ... | 8·32 |

appear in the preceding paper [1928]. It is there suggested that although Folin's method may not be reliable as an absolute measurement of the amino-acids in a given sample of blood, a series of results are not rendered nugatory provided they are carried out under the same conditions, and preferably by the same worker. If these precautions are taken, figures in such a series, considered relatively, are quite trustworthy.

Since our own experiments fulfilled these conditions, a comparison of the amino-acid-nitrogen results for the chief diseases investigated can now be undertaken.

## Blood-amino-acid in disease.

### *Rheumatic carditis, chorea, etc.*

The total estimations carried out upon rheumatic carditis with and without chorea, on chorea itself, and on acute rheumatism number 52. Of these, 32 were taken fasting, the remainder non-fasting.

The mean of the fasting results was 8·21 mg. %. This, compared with the fasting normal of 8·32, does not differ sufficiently to be of importance. The non-fasting mean was 9·15 mg. %.

We therefore consider that the amino-acid-content of rheumatic bloods is normal.

### *Nephritis.*

Twenty-five cases of nephritis were examined. Of these, 21 were taken fasting and 4 non-fasting.

Only 8 separate patients were examined of the fasting group, some of them yielding several samples. On the whole the results were normal, tending if anything to become slightly raised for acute haemorrhagic nephritis, of which several cases were examined. An exception was R. L., a boy of 10 years, suffering from a severe mixed nephritis, who yielded 8 specimens. The highest of the figures was 8·14 mg. %, the lowest 5·13, and the mean 6·58.

*Renal infantilism and coeliac infantilism.*

Cases of these less common diseases were examined from time to time. Although the chief interest in their blood chemistry centres around the calcium and phosphorus content, it will be seen that the amino-acids are also abnormal.

Seventeen cases of renal infantilism and 6 coeliac cases were examined. The mean of the renals was 7·06 and of the coeliacs 7·28 mg. %.

In Table III are summarised the "fasting" results discussed above.

Table III.

| Diagnosis | No. of estimations | Amino-acid-N mg. % | | |
|---|---|---|---|---|
| | | Max. | Min. | Mean |
| Normal | 16 | 9·52 | 6·08 | 8·32 |
| Rheumatism, etc. | 32 | 10·07 | 5·69 | 8·21 |
| Nephritis: | | | | |
| (*a*) Ac. haemorrhagic | 11 | 10·20 | 7·65 | 8·48 |
| (*b*) Mixed (R.L.) | 8 | 8·14 | 5·13 | 6·58 |
| (*c*) Chronic | 1 | — | — | 8·48 |
| (*d*) Pyelo-nephritis | 1 | — | — | 8·70 |
| Renal infantilism | 17 | 9·52 | 5·02 | 7·06 |
| Coeliac infantilism | 6 | 8·07 | 6·09 | 7·28 |

SUMMARY.

Estimations of the blood amino-acid-nitrogen in normal children and in those in various pathological conditions, both fasting and non-fasting, are reported.

The amino-acid-nitrogen has consistently been found to be lower in fasting bloods than in non-fasting, and the mean difference due to this influence is estimated.

The blood amino-acid-nitrogen for normal healthy children has been determined, and by comparison with this figure it has been shown that:

(*a*) in rheumatism and chorea with or without carditis no change is found;

(*b*) in nephritis there is a lowering of the amino-nitrogen;

(*c*) in renal and coeliac infantilism this lowering is still more marked.

The author wishes to express his thanks to Dr L. G. Parsons, physician, and Dr E. M. Hickmans, biochemist, of the Children's Hospital, Birmingham, for their help and interest in the foregoing research; to the members of the resident staff who so readily assisted in the collection of blood samples during the course of the work; and to the Medical Research Council for a grant which has helped to defray expenses.

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# XXV. THE INFLUENCE OF THE COW'S DIET ON THE FAT-SOLUBLE VITAMINS OF WINTER MILK. II.

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In a previous communication [Golding, Soames and Zilva, 1926] it was shown that by administering cod-liver oil to cows kept on a diet rigorously restricted in the fat-soluble vitamins during the winter, the vitamin A and D content of the butter was greatly enhanced. On the other hand, when this basal diet was supplemented by kale there was an increase in the vitamin A content of the butter but its antirachitic value remained unaltered. These experiments were instituted with the definite purpose of demonstrating the differential influence of the feeding of cod-liver oil and of kale to cows during the winter on the antirachitic potency of the butter, and consequently extreme diets were aimed at independently of their practical applicability. Thus the basal diet alone was much more drastically deficient in vitamins than is usual with the winter ration of cows employed in this country. Moreover, the dose of cod-liver oil administered was of such a magnitude as to depress the fat content of the milk [Drummond *et al.*, 1924; Channon *et al.*, 1924].

Table I.

| | | No. of calves | Date last calved |
|---|---|---|---|
| Rigorous basal diet | Gertie II | 1 | Oct. 31, 1926 |
| | Rufa III | 1 | Sept. 6, ,, |
| Control basal diet | Flora II | 2 | Dec. 28, ,, |
| | Clara I | 1 | Nov. 23, ,, |
| Basal diet + cod-liver oil to excess | Winsome II | 1 | ,, 30, ,, |
| | Flora I | 3 | ,, 16, ,, |
| Basal diet + 2 oz. cod-liver oil | Lily II | 2 | Dec. 1, ,, |
| | Doll I | 8 | Nov. 8, ,, |

This communication deals with the influence of doses of cod-liver oil, which are not high enough to depress the fat content of the milk, on the vitamin D potency of butter, and also with the antirachitic value of a good ration such as is frequently given to stall-fed cows during the winter in England.

The data recorded will be mainly those which have a bearing on the antirachitic value of the butter and on the depression of the milk-fat due to the administration of cod-liver oil. Other data are reserved for future record.

## Table II. *Weights of food given during experimental period.*

| Name of cow ... ... ... ... | Flora I | Winsome II | Doll I | Lily II | Clara I | Flora II |
|---|---|---|---|---|---|---|
| Dates of experimental period for each cow | Dec. 6, 1926 to March 28, 1927 | | | | | Jan. 18 to Mar. 23, 1927 |
| Food — Date | lb. | lb. | lb. | lb. | lb. | lb. |
| Cabbage — Dec. 1–31 | 40 | 40 | 40 | 40 | 40 | 40 |
| Silage — Nov. 1–Dec. 31 | 15 | 15 | 15 | 15 | 15 | 15 |
| Jan. 1–Mar. 5 | 25 | 25 | 25 | 25 | 25 | 25 |
| Mar. 6–18 | 40 | 40 | 40 | 40 | 40 | 40 |
| „ 19–28 | 30 | 30 | 30 | 30 | 30 | 30 |
| Mangolds — Nov. 1–Mar. 5 | 28 | 28 | 28 | 28 | 28 | 28 |
| Hay — „ 1–Apr. 6 | 9 | 9 | 9 | 9 | 9 | 9 |

Mixtures

| Concentrates — From | A in lb. | B in lb. | C.L.O. in oz. | A in lb. | B in lb. | C.L.O. in oz. | A in lb. | B. in lb. | C.L.O. in oz. | A in lb. | B in lb. | C.L.O. in oz. | A in lb. | B in lb. | Arach. oil in oz. | A in lb. | B in lb. | Arach. oil in oz. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Dec. 1 | 10 | 2 | — | — | — | — | 10 | 8 | — | — | — | — | 8 | — | — | — | — | — |
| „ 2 | „ | 4 | — | — | — | — | „ | „ | — | — | — | — | 10 | — | — | — | — | — |
| „ 3 | „ | 6 | — | — | — | — | „ | „ | — | — | — | — | „ | — | — | — | — | — |
| „ 4 | „ | „ | — | 3 | — | — | „ | „ | — | — | — | — | „ | — | — | — | — | — |
| „ 5 | „ | „ | — | 6 | 2 | — | „ | „ | — | 3 | — | — | „ | — | — | — | — | — |
| „ 6 | „ | 8 | — | „ | „ | — | „ | „ | — | 6 | — | — | „ | 2 | — | — | — | — |
| „ 7 | „ | „ | — | 8 | „ | — | „ | „ | — | „ | — | — | „ | „ | — | — | — | — |
| „ 8 | „ | „ | — | „ | „ | — | „ | „ | — | 8 | — | — | „ | „ | — | — | — | — |
| „ 9 | „ | „ | — | 10 | „ | — | „ | „ | — | „ | — | — | „ | „ | — | — | — | — |
| „ 25 | „ | „ | — | „ | „ | — | „ | „ | — | 9 | — | — | „ | „ | — | — | — | — |
| „ 26 | „ | „ | — | „ | „ | — | „ | „ | — | 10 | — | — | „ | „ | — | — | — | — |
| „ 29 | „ | „ | — | „ | „ | — | „ | „ | — | „ | 2 | — | „ | „ | — | — | — | — |
| Jan. 1 | „ | „ | — | „ | „ | — | „ | „ | — | „ | „ | — | „ | „ | — | 3 | — | — |
| „ 2 | „ | „ | — | „ | „ | — | „ | „ | — | „ | „ | — | „ | „ | — | 6 | — | — |
| „ 4 | „ | „ | — | „ | „ | — | „ | „ | — | „ | „ | — | „ | „ | — | 8 | — | — |
| „ 5 | „ | „ | — | „ | „ | — | „ | „ | — | „ | „ | — | „ | „ | — | 10 | — | — |
| „ 6 | „ | „ | 2 | „ | 3 | ½ | „ | „ | 2 | „ | „ | ½ | „ | 3 | — | „ | — | — |
| „ 7 | „ | „ | „ | „ | „ | „ | „ | „ | „ | „ | „ | „ | „ | „ | — | „ | 2 | — |
| „ 8 | „ | „ | „ | „ | „ | 2 | „ | „ | „ | „ | „ | 2 | „ | „ | — | „ | „ | — |
| „ 10 | „ | „ | „ | „ | „ | „ | „ | „ | „ | „ | 4 | „ | „ | „ | — | „ | 4 | — |
| „ 11 | „ | „ | 4 | „ | „ | 4 | „ | „ | „ | „ | „ | „ | „ | „ | — | „ | „ | — |
| „ 21 | „ | „ | „ | „ | „ | „ | „ | „ | „ | „ | „ | „ | „ | „ | — | „ | 6 | — |
| „ 29 | „ | „ | 6 | „ | „ | 6 | „ | „ | „ | „ | „ | „ | „ | „ | — | „ | „ | — |
| Feb. 10 | „ | 6 | „ | „ | „ | „ | „ | 6 | „ | „ | „ | „ | „ | „ | — | „ | 8 | — |
| „ 12 | „ | 5 | „ | „ | „ | „ | „ | 4 | „ | „ | „ | — | „ | „ | 2 | „ | „ | 2 |
| „ 20 | „ | „ | „ | „ | 2 | „ | „ | „ | „ | „ | 3 | „ | „ | „ | „ | „ | „ | „ |
| „ 26 | „ | „ | 8 | „ | „ | „ | „ | „ | „ | „ | „ | „ | „ | „ | 4 | „ | „ | 4 |
| Mar. 6 | „ | „ | „ | „ | „ | „ | „ | „ | „ | „ | „ | „ | „ | — | 6 | „ | „ | 6 |
| „ 13 | „ | „ | „ | „ | „ | „ | „ | „ | „ | „ | „ | „ | „ | „ | 8 | „ | „ | 8 |
| „ 28 | „ | „ | „ | „ | „ | „ | „ | „ | „ | 6 | „ | * | „ | „ | * | 8 | „ | * |

* Last dose Mar. 28.

Mixture A:

| | |
|---|---|
| Palm kernel meal extracted ... | 2 parts |
| French maize gluten feed ... | 2 „ |
| Rice meal ... ... ... ... | 2 „ |
| Undecorticated ground nut meal ext. | 1 part |
| Decorticated ground nut meal ext. | 1 „ |
| Crushed oats ... ... ... | 1 „ |

Mixture B:

| | |
|---|---|
| Decorticated ground nut cake | 1 part |
| B.B. and O. mixture ... ... | 3 parts |
| Maize germ cake ... ... | 2 „ |

B.B. and O. mixture. Balanced for milk production

| | |
|---|---|
| Decorticated ground nut cake | |
| Decorticated cotton seed meal | |
| Palm kernel cake | |
| Maize gluten feed | |
| Rangoon rice meal | |
| Essential mineral matter | |

## Experimental.

The conditions under which these experiments were carried out were essentially the same as those described in the first communication of this series. Eight dairy Shorthorns were divided into four groups, as described in Table I, which also gives the history of the animals, whilst Table II gives the diet of six of these animals during the experimental period. It will be seen that the control cows, Flora II and Clara I, received a good winter stall ration. Gertie II and Rufa III, on the other hand, were given a very restricted diet, such as would seldom be employed in practice in England. It was made up as follows:

| | |
|---|---|
| Bean meal ... ... ... ... ... | 2 parts |
| Extracted palm kernel meal ... ... | 2 ,, |
| Decorticated ground nut meal ... ... | 1 part |
| Barley meal ... ... ... ... ... | 1 ,, |
| Crushed wheat ... ... ... ... | 1 ,, |
| Oat straw ... ... ... ... ... | *ad lib.* |
| Roots ... ... ... ... ... ... | 50 lb. |

This diet was started on January 18, 1927.

From October 21 the cows were kept in during the night but were turned out to grass during the mornings, except Gertie II and Rufa III which were stalled during the entire 24 hours from Jan. 18th. The administration of the cod-liver oil began in the first week in January. In the case of Flora I the dose was gradually increased to 8 oz. and in that of Winsome II to 6 oz. Doll I and Lily II received 2 oz. *per diem* through the entire experimental period.

In order to observe the influence of the various doses of cod-liver oil on the fat content, the milk of each cow was weighed, mixed and sampled at each milking and the fat content determined by the Gerber method.

The butters were churned for testing purposes during the second fortnight in March and Table III gives the details of the churnings.

The antirachitic vitamin content of the butters was determined by the method previously described. Owing to exigencies of circumstances, it was not found convenient to test the butters all together, and consequently the positive and negative control groups of animals are those of the particular groups amongst which the respective samples were tested. The milks of Flora I and Winsome II were mixed together and the butter prepared from this mixed sample was tested.

## Discussion of results.

The object of this investigation was to ascertain whether it was possible to feed to cows doses of cod-liver oil of such a magnitude that they would not depress the milk-fat but would raise the antirachitic value of the milk.

Table III. *Weight of milk, percentage of fat, weight of fat, percentage of solids not fat, and actual weight of butter made from which samples were taken to the Lister Institute on April* 27, 1927.

| Cow | Milk taken for butter | Wt of milk lb. | % fat | Wt of fat lb. | Solids not fat % | Time of churning and wt of butter | |
|---|---|---|---|---|---|---|---|
| | | | | | | min. | lb. oz. |
| Flora I | Mar. 17, p.m. | 13 | 3·75 | 0·487 | | | |
| | „ 18 „ | 14¾ | 4·15 | 0·612 | | | |
| Winsome II | „ 17 „ | 9¼ | 4·50 | 0·416 | | | |
| | „ 18 „ | 9 | 4·15 | 0·373 | | | |
| Flora I } milks | „ 17 „ | 22¼ | 4·05 | 0·801 | | | |
| Winsome II } mixed | „ 18 „ | 23¾ | 4·20 | 0·997 | — | 45 | 1 6 |
| Doll I | „ 23, p.m. | 13½ | 4·2 | 0·567 | | | |
| | „ 24, a.m. | 16 | 4·2 | 0·672 | | | |
| | „ 24, p.m. | 13½ | 4·85 | 0·655 | 8·95 | | |
| | „ 25, a.m. | 15¾ | 4·0 | 0·630 | 8·56 | 35 | 2 8 |
| Lily II | „ 23, p.m. | 11¼ | 3·8 | 0·427 | | | |
| | „ 24, a.m. | 17¾ | 3·0 | 0·532 | | | |
| | „ 24, p.m. | 10¼ | 4·0 | 0·210 | 9·03 | | |
| | „ 25, a.m. | 16¾ | 2·75 | 0·460 | 8·83 | 25 | 1 15 |
| Flora II | „ 21, a.m. | 27 | 2·85 | 0·769 | | | |
| | „ 21, p.m. | 20 | 3·95 | 0·790 | 8·85 | | |
| | „ 22, a.m. | 27 | 3·10 | 0·837 | 8·88 | | |
| | „ 22, p.m. | 19¼ | 4·05 | 0·778 | | | |
| | „ 23, a.m. | 26¾ | 2·70 | 0·722 | — | 37 | 3 8 |
| Clara I | „ 21, a.m. | 18 | 2·95 | 0·531 | | | |
| | „ 21, p.m. | 10½ | 5·4 | 0·567 | 9·02 | | |
| | „ 22, a.m. | 17½ | 3·0 | 0·525 | 9·13 | | |
| | „ 22, p.m. | 10½ | 4·95 | 0·519 | | | |
| | „ 23, a.m. | 18 | 3·20 | 0·576 | — | 35 | 2 8 |
| Gertie II | „ 19, a.m. | 11¼ | | | | | |
| | „ 19, p.m. | 6½ | | | | | |
| | „ 20, a.m. | 11½ | | | | | |
| | „ 20, p.m. | 6½ | | | | | |
| | „ 21, a.m. | 11½ | — | — | — | 17 | 1 12 |
| Rufa III | „ 19, a.m. | 11½ | | | | | |
| | „ 19, p.m. | 6¼ | 4·3 | | | | |
| | „ 20, a.m. | 12 | | | | | |
| | „ 20, p.m. | 6 | | | | | |
| | „ 21, a.m. | 11¾ | — | — | — | 95 | 1 10 |

The results, however, are not as clear-cut as might be desired, and further work of a confirmatory character is called for, which it is hoped will be forthcoming in connection with a wider inquiry in which the authors are now engaged.

It is seen from Fig. 1 that the curves of the average weekly percentage of fat in the milk of Flora II and Clara, which received a good winter ration, but no cod-liver oil, are nearly horizontal lines, at the level of about 4 %. In the case of Winsome II, receiving a high dose of cod-liver oil, there is a decided progressive drop in the percentage of fat until about the first week in February, when the increase of the dose to 6 oz. produced no further decrease in the percentage of the fat. The effect of the administration of similar doses of cod-liver oil to the companion cow of this group, Flora I, was less marked. When we turn our attention to the cows which received a daily dose of 2 oz. of cod-liver oil, we find that, if there be a depression in the percentage of the milk-fat, it is of a very much lower order than in the

cases of Winsome II and Flora I. The curve for Doll I is very similar to that of the control cows, Clara and Flora II. Lily II, on the other hand, shows a depression, but it will be noticed that it begins about four weeks before

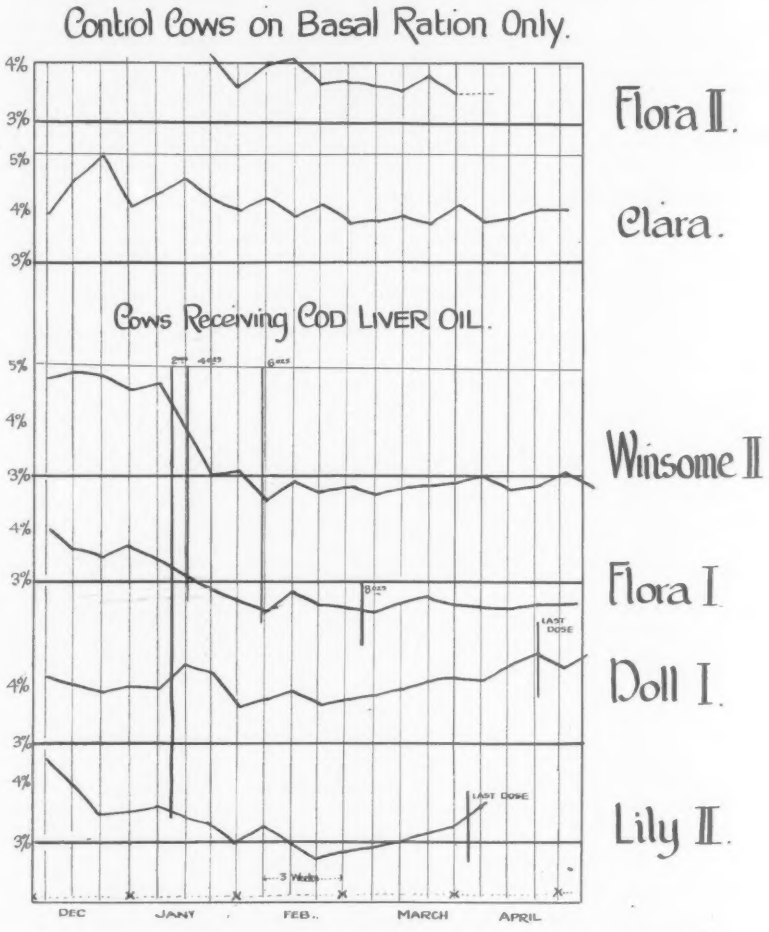


Fig. 1. Chart indicating the effect of varying doses of cod-liver oil in depressing the average weekly percentage of fat in the milk of cows—Dec. 1926–May 1927.

the administration of the cod-liver oil began and persists until about the middle of the cod-liver oil period, when a gradual and sustained return to the normal begins. We are not inclined to attribute this depression to the influence of the oil.

Table IV.

| Rat | Initial weight g. | Final weight g. | Weight of bone g. | % of water | % of fat | % of organic residue | % of ash | Ratio *A/R* | Average ratio *A/R* |
|---|---|---|---|---|---|---|---|---|---|
| *Positive controls for Winsome and Flora I (mixed) and Clara and Flora II* | | | | | | | | | |
| Daily dose—3 drops of cod-liver oil | | | | | | | | | |
| 5919 ♀ | 50 | 74 | 1·1041 | 51·40 | 6·81 | 21·13 | 20·67 | 0·9779 | |
| 5920 ♂ | 50 | 69 | 1·1638 | 54·33 | 2·48 | 22·77 | 20·88 | 0·9170 | |
| 5921 ♂ | 50 | 77 | 1·1566 | 58·99 | 2·32 | 23·08 | 18·26 | 0·7911 | |
| 5922 ♀ | 50 | 74 | 1·0479 | 49·91 | 4·39 | 23·59 | 22·09 | 0·9361 | |
| 5923 ♂ | 50 | 63 | 1·0199 | 55·99 | 2·53 | 22·06 | 20·04 | 0·9084 | |
| 5924 ♂ | 50 | 75 | 1·0511 | 50·03 | 2·19 | 23·27 | 24·52 | 1·0540 | |
| 5925 ♀ | 50 | 57 | 0·9712 | 56·20 | 3·11 | 22·17 | 18·51 | 0·8354 | |
| 5926 ♂ | 50 | 74 | 1·1742 | 55·01 | 1·50 | 23·09 | 20·44 | 0·8853 | 0·9132 |
| *Negative controls for Winsome and Flora I (mixed) and Clara and Flora II* | | | | | | | | | |
| 5911 ♀ | 50 | 59 | 0·9409 | 58·86 | 5·08 | 22·05 | 14·04 | 0·6370 | |
| 5912 ♂ | 50 | 84 | 1·1012 | 59·09 | 5·32 | 21·41 | 14·20 | 0·6633 | |
| 5914 ♂ | 50 | 78 | 1·1582 | 62·43 | 2·98 | 21·35 | 13·24 | 0·6184 | |
| 5915 ♂ | 50 | 86 | 1·2851 | 63·87 | 2·54 | 21·88 | 11·72 | 0·5357 | |
| 5916 ♂ | 50 | 79 | 1·1373 | 60·68 | 2·22 | 22·86 | 13·72 | 0·6002 | |
| 5917 ♀ | 50 | 76 | 1·1940 | 65·16 | 2·51 | 22·82 | 9·51 | 0·4168 | |
| 5918 ♂ | 50 | 74 | 1·0853 | 60·91 | 3·12 | 24·40 | 11·58 | 0·4710 | 0·5632 |
| 6–8 *oz. cod-liver oil* (*Winsome and Flora I mixed*) | | | | | | | | | |
| Daily dose—0·2 g. butter | | | | | | | | | |
| 5899 ♀ | 50 | 70 | 1·1039 | 60·29 | 1·67 | 22·56 | 16·39 | 0·7269 | |
| 5900 ♂ | 50 | 84 | 1·2262 | 58·93 | 4·83 | 21·20 | 15·03 | 0·7087 | |
| 5901 ♂ | 50 | 76 | 0·9960 | 63·17 | 2·28 | 24·32 | 10·24 | 0·4211 | |
| 5902 ♀ | 50 | 75 | 1·1054 | 54·24 | 3·87 | 22·64 | 19·33 | 0·8540 | |
| 5903 ♀ | 50 | 82 | 1·0403 | 52·70 | 2·80 | 24·47 | 19·94 | 0·8131 | |
| 5904 ♂ | 50 | 70 | 1·1295 | 54·25 | 7·74 | 20·53 | 17·44 | 0·8494 | 0·7289 |
| Daily dose—0·6 g. butter | | | | | | | | | |
| 5905 ♂ | 50 | 88 | 1·1933 | 60·05 | 1·70 | 22·80 | 15·49 | 0·6794 | |
| 5906 ♂ | 50 | 70 | 1·1357 | 58·09 | 2·38 | 23·08 | 17·29 | 0·7491 | |
| 5907 ♀ | 50 | 75 | 1·0048 | 52·34 | 1·68 | 23·63 | 22·34 | 0·9454 | |
| 5908 ♂ | 50 | 80 | 1·1966 | 54·45 | 3·87 | 21·68 | 19·98 | 0·9216 | |
| 5909 ♂ | 50 | 74 | 1·1474 | 49·68 | 0·97 | 20·28 | 20·40 | 1·0060 | |
| 5910 ♀ | 50 | 86 | 1·2115 | 52·17 | 4·48 | 22·62 | 20·70 | 0·9151 | 0·8694 |
| *Good winter ration* (*Clara*) | | | | | | | | | |
| Daily dose—0·2 g. butter | | | | | | | | | |
| 5927 ♀ | 50 | 79 | 1·1049 | 63·36 | 2·10 | 14·48 | 10·91 | 0·7536 | |
| 5928 ♀ | 50 | 72 | 0·9815 | 57·58 | 3·48 | 22·72 | 16·19 | 0·7126 | |
| 5930 ♀ | 50 | 79 | 0·9509 | 60·52 | 3·62 | 21·81 | 14·06 | 0·6447 | |
| 5931 ♂ | 50 | 72 | 1·0610 | 60·82 | 2·55 | 21·68 | 14·96 | 0·6900 | |
| 5932 ♂ | 50 | 80 | 1·1331 | 61·20 | 4·07 | 21·84 | 12·87 | 0·5893 | 0·6780 |
| Daily dose—0·6 g. butter | | | | | | | | | |
| 5933 ♂ | 47 | 71 | 0·9939 | 58·06 | 2·99 | 21·55 | 17·41 | 0·8076 | |
| 5934 ♂ | 50 | 90 | 1·1500 | 60·84 | 2·82 | 21·75 | 14·59 | 0·6710 | |
| 5935 ♀ | 50 | 70 | 0·9286 | 59·78 | 3·22 | 22·09 | 14·91 | 0·6749 | |
| 5936 ♂ | 50 | 68 | 1·1021 | 57·31 | 1·74 | 21·71 | 19·17 | 0·8830 | |
| 5937 ♀ | 50 | 85 | 1·0451 | 59·51 | 2·19 | 22·43 | 16·29 | 0·7258 | |
| 5938 ♂ | 50 | 86 | 1·1729 | 56·45 | 4·71 | 21·44 | 17·40 | 0·8110 | 0·7622 |
| *Good winter ration* (*Flora II*) | | | | | | | | | |
| Daily dose—0·2 g. butter | | | | | | | | | |
| 5939 ♀ | 50 | 78 | 1·0825 | 63·46 | 2·58 | 22·25 | 11·69 | 0·5254 | |
| 5940 ♀ | 50 | 72 | 0·9781 | 61·98 | 2·59 | 22·24 | 13·19 | 0·5937 | |
| 5941 ♀ | 50 | 85 | 1·1384 | 53·52 | 3·79 | 22·06 | 20·66 | 0·9369 | |
| 5942 | 50 | 73 | 1·0223 | 63·45 | 2·79 | 22·30 | 11·49 | 0·5152 | |
| 5943 | 50 | 86 | 1·3120 | 59·87 | 4·94 | 21·59 | 13·59 | 0·6295 | 0·6401 |

Table IV (*cont.*)

| Rat | Initial weight g. | Final weight g. | Weight of bone g. | % of water | % of fat | % of organic residue | % of ash | Ratio *A*/*R* | Average ratio *A*/*R* |
|---|---|---|---|---|---|---|---|---|---|
| *Good winter ration (Flora II)* | | | | | | | | | |
| Daily dose—0·6 g. butter | | | | | | | | | |
| 5944 ♀ | 50 | 84 | 1·0925 | 56·06 | 1·07 | 22·01 | 19·54 | 0·8877 | |
| 5945 ♀ | 50 | 80 | 1·1217 | 53·44 | 3·94 | 22·12 | 20·48 | 0·9259 | |
| 5946 ♂ | 50 | 98 | 1·3468 | 60·60 | 2·80 | 22·03 | 14·84 | 0·6736 | |
| 5947 ♀ | 50 | 74 | 1·0589 | 57·89 | 3·07 | 22·13 | 16·92 | 0·7650 | |
| 5948 ♀ | 50 | 72 | 1·0268 | 55·54 | 3·51 | 21·96 | 19·04 | 0·8670 | |
| 5949 ♀ | 50 | 78 | 1·1106 | 61·75 | 3·30 | 22·59 | 12·34 | 0·5462 | 0·7776 |
| *Positive controls for Lily and Gertie* | | | | | | | | | |
| 5846 ♀ | 33 | 57 | 0·8783 | 54·91 | 3·77 | 21·75 | 19·58 | 0·9005 | |
| 5849 ♀ | 32 | 52 | 0·7545 | 54·33 | 5·69 | 21·40 | 18·49 | 0·8632 | |
| 5850 ♀ | 30 | 50 | 0·7696 | 55·05 | 7·93 | 18·75 | 18·28 | 0·9802 | 0·9146 |
| *Negative controls for Lily and Gertie* | | | | | | | | | |
| 5851 ♂ | 32 | 47 | 0·8547 | 64·36 | 4·95 | 21·41 | 9·28 | 0·4333 | |
| 5852 ♀ | 30 | 42 | 0·7012 | 63·36 | 3·18 | 22·88 | 10·59 | 0·4640 | |
| 5853 ♀ | 35 | 50 | 0·7784 | 58·98 | 4·50 | 23·64 | 13·17 | 0·5572 | |
| 5854 ♀ | 35 | 54 | 0·7170 | 60·63 | 5·24 | 21·51 | 12·12 | 0·5635 | |
| 5855 ♀ | 33 | 47 | 0·6826 | 62·82 | 3·97 | 22·63 | 10·56 | 0·4667 | |
| 5856 ♀ | 30 | 48 | 0·8262 | 65·73 | 2·59 | 22·49 | 9·19 | 0·4085 | |
| 5857 ♀ | 35 | 53 | 0·7997 | 61·87 | 2·18 | 24·03 | 11·92 | 0·4960 | |
| 5858 ♀ | 37 | 57 | 0·9641 | 63·27 | 3·34 | 22·91 | 10·47 | 0·4590 | 0·4810 |
| 2 *oz. cod-liver oil* (*Lily*) | | | | | | | | | |
| Daily dose—0·2 g. butter | | | | | | | | | |
| 5819 ♂ | 50 | 80 | 1·2600 | 63·36 | 3·50 | 22·24 | 10·90 | 0·4904 | |
| 5820 ♂ | 30 | 58 | 0·8623 | 64·83 | 3·17 | 20·81 | 11·21 | 0·5389 | |
| 5821 ♂ | 37 | 67 | 0·9975 | 59·55 | 0·09 | 23·97 | 15·56 | 0·6498 | |
| 5822 ♀ | 47 | 75 | 0·9728 | 58·34 | 5·93 | 24·69 | 13·55 | 0·4463 | |
| 5824 ♀ | 46 | 63 | 0·9072 | 62·79 | 3·51 | 23·48 | 10·23 | 0·4356 | 0·5122 |
| Daily dose—0·6 g. butter | | | | | | | | | |
| 5825 ♀ | 30 | 54 | 0·8838 | 57·57 | 4·37 | 20·90 | 9·14 | 0·8206 | |
| 5826 ♂ | 32 | 59 | 0·7978 | 57·61 | 6·05 | 21·51 | 14·83 | 0·6895 | |
| 5827 ♀ | 36 | 67 | 0·9370 | 57·82 | 4·23 | 22·54 | 20·42 | 0·9055 | |
| 5828 ♀ | 36 | 65 | 1·0422 | 62·91 | 1·74 | 20·25 | 15·11 | 0·7464 | |
| 5829 ♀ | 34 | 65 | 0·8597 | 61·83 | 2·26 | 21·83 | 14·07 | 0·6448 | |
| 5830 ♂ | 32 | 59 | 0·9406 | 57·90 | 4·02 | 21·11 | 17·02 | 0·8063 | 0·7689 |
| *Very restricted diet* (*Gertie*) | | | | | | | | | |
| Daily dose—0·2 g. butter | | | | | | | | | |
| 5831 ♂ | 33 | 59 | 0·8825 | 64·22 | 3·37 | 22·45 | 9·91 | 0·4417 | |
| 5832 ♀ | 37 | 62 | 0·8583 | 63·68 | 2·40 | 22·42 | 11·50 | 0·5130 | |
| 5833 ♂ | 36 | 62 | 0·8890 | 63·11 | 2·36 | 22·80 | 11·73 | 0·5144 | |
| 5834 ♀ | 42 | 63 | 0·9340 | 61·81 | 3·44 | 22·62 | 11·57 | 0·5117 | |
| 5835 ♀ | 30 | 50 | 0·8045 | 64·33 | 2·60 | 22·70 | 10·36 | 0·4567 | |
| 5836 ♂ | 35 | 60 | 0·8528 | 65·59 | 3·76 | 21·98 | 9·70 | 0·4414 | 0·4798 |
| Daily dose—0·6 g. butter | | | | | | | | | |
| 5837 ♀ | 33 | 51 | 0·7397 | 64·45 | 3·26 | 22·94 | 9·37 | 0·4085 | |
| 5838 ♂ | 34 | 57 | 0·9792 | 64·66 | 3·87 | 21·65 | 9·82 | 0·4533 | |
| 5839 ♀ | 33 | 53 | 0·9139 | 59·91 | 7·39 | 20·85 | 11·95 | 0·5738 | |
| 5840 ♂ | 34 | 63 | 0·9233 | 55·75 | 4·28 | 21·52 | 17·97 | 0·8351 | |
| 5841 ♀ | 33 | 69 | 0·9304 | 61·52 | 4·86 | 22·25 | 11·37 | 0·5110 | |
| 5842 ♂ | 40 | 68 | 1·0986 | 61·89 | 3·98 | 21·97 | 12·14 | 0·5524 | 0·5557 |
| *Positive controls for Rufa and Doll* | | | | | | | | | |
| Daily dose—1 drop cod-liver oil | | | | | | | | | |
| 5883 ♀ | 40 | 70 | 1·0226 | 50·23 | 8·18 | 21·00 | 20·55 | 0·9786 | |
| 5884 ♂ | 48 | 55 | 0·9439 | 51·87 | 3·91 | 21·94 | 21·09 | 0·9620 | |
| 5885 ♂ | 33 | 65 | 0·8673 | 51·76 | 7·89 | 21·25 | 19·08 | 0·8978 | |
| 5886 ♀ | 48 | 75 | 1·1396 | 52·36 | 5·94 | 20·96 | 20·71 | 0·9880 | 0·9566 |

Table IV (*cont.*)

| Rat | Initial weight g. | Final weight g. | Weight of bone g. | % of water | % of fat | % of organic residue | % of ash | Ratio *A/R* | Average ratio *A/R* |
|---|---|---|---|---|---|---|---|---|---|
| | | | *Positive controls for Rufa and Doll* | | | | | | |
| | | | Daily dose—3 drops cod-liver oil | | | | | | |
| 5887 ♀ | 30 | 60 | 1·0029 | 53·55 | 1·75 | 20·22 | 18·73 | 0·9263 | |
| 5888 ♂ | 35 | 55 | 0·8721 | 51·36 | 8·40 | 21·37 | 18·79 | 0·8792 | |
| 5889 ♂ | 36 | 60 | 0·9006 | 50·18 | 6·65 | 22·38 | 20·81 | 0·9298 | |
| 5890 ♀ | 33 | 55 | 0·7598 | 47·63 | 8·16 | 21·01 | 19·79 | 0·9419 | 0·9183 |
| | | | *Negative controls for Rufa and Doll* | | | | | | |
| 5893 ♂ | 43 | 53 | 0·8814 | 55·97 | 7·16 | 22·28 | 14·59 | 0·6548 | |
| 5894 ♂ | 45 | 65 | 0·9260 | 59·88 | 6·56 | 21·93 | 11·64 | 0·5308 | |
| 5895 ♀ | 33 | 60 | 0·8237 | 60·87 | 3·11 | 23·19 | 12·81 | 0·5524 | |
| 5896 ♀ | 35 | 70 | 0·9838 | 62·40 | 5·44 | 22·06 | 10·09 | 0·4574 | |
| 5897 ♂ | 42 | 55 | 0·7669 | 55·04 | 7·41 | 22·41 | 15·15 | 0·6760 | |
| 5898 ♂ | 32 | 55 | 0·8130 | 56·72 | 7·88 | 21·03 | 14·35 | 0·6824 | 0·5923 |
| | | | *Very restricted diet (Rufa)* | | | | | | |
| | | | Daily dose—0·2 g. butter | | | | | | |
| 5859 ♀ | 42 | 47 | 0·8123 | 65·51 | 1·01 | 23·96 | 9·49 | 0·3961 | |
| 5860 ♂ | 42 | 60 | 0·8695 | 63·90 | 3·22 | 22·67 | 10·09 | 0·4450 | |
| 5861 ♂ | 45 | 67 | 1·0434 | 64·54 | 9·49 | 24·99 | 9·56 | 0·3825 | |
| 5862 ♀ | 43 | 65 | 0·9922 | 58·86 | 3·02 | 23·23 | 15·09 | 0·6496 | |
| 5863 ♀ | 43 | 63 | 1·1461 | 70·84 | 2·09 | 19·81 | 9·15 | 0·4618 | |
| 5864 ♀ | 38 | 45 | 0·8451 | 55·52 | 3·62 | 24·19 | 16·65 | 0·6878 | 0·5039 |
| | | | Daily dose—0·6 g. butter | | | | | | |
| 5865 ♀ | 44 | 60 | 0·9065 | 62·70 | 3·73 | 22·07 | 11·51 | 0·5215 | |
| 5866 ♀ | 36 | 60 | 0·8876 | 61·35 | 3·62 | 21·96 | 13·07 | 0·5952 | |
| 5867 ♂ | 48 | 70 | 1·0601 | 55·14 | 6·09 | 20·92 | 17·86 | 0·8537 | |
| 5868 ♂ | 44 | 70 | 0·9524 | 62·63 | 0·95 | 24·60 | 11·82 | 0·4806 | |
| 5869 ♂ | 40 | 60 | 0·9424 | 60·03 | 3·00 | 23·35 | 13·58 | 0·5815 | |
| 5870 ♀ | 38 | 59 | 0·7954 | 59·19 | 4·73 | 21·79 | 14·29 | 0·6559 | 0·6147 |
| | | | *2 oz. cod-liver oil (Doll)* | | | | | | |
| | | | Daily dose—0·2 g. butter | | | | | | |
| 5871 ♂ | 40 | 65 | 0·9515 | 55·95 | 2·90 | 23·36 | 17·88 | 0·7654 | |
| 5872 ♀ | 43 | 55 | 0·8668 | 63·97 | 1·29 | 22·32 | 13·15 | 0·5849 | |
| 5873 ♂ | 43 | 65 | 1·1204 | 64·04 | 1·80 | 21·97 | 12·21 | 0·5560 | |
| 5874 ♀ | 35 | 60 | 0·9592 | 57·57 | 4·09 | 21·21 | 17·12 | 0·8071 | |
| 5876 ♀ | 37 | 55 | 0·8505 | — | — | 22·76 | 16·23 | 0·7131 | 0·6853 |
| | | | Daily dose—0·6 g. butter | | | | | | |
| 5877 ♀ | 36 | 55 | 0·8627 | 53·83 | 5·55 | 21·92 | 18·69 | 0·8527 | |
| 5878 ♂ | 48 | 60 | 1·0690 | 51·29 | 9·74 | 21·10 | 17·89 | 0·8480 | |
| 5879 ♂ | 37 | 63 | 0·9482 | 56·82 | 2·07 | 23·44 | 17·69 | 0·7547 | |
| 5880 ♀ | 50 | 75 | 0·9480 | 53·71 | 6·55 | 20·39 | 19·34 | 0·9485 | |
| 5881 ♂ | 35 | 60 | 1·0774 | 55·89 | 8·41 | 19·80 | 15·95 | 0·8055 | |
| 5882 ♂ | 37 | 60 | 0·9451 | 56·83 | 6·66 | 20·77 | 15·74 | 0·7583 | 0·8279 |

The results of the antirachitic tests (Table IV and Fig. 2) do not reveal any striking superiority of the butters from Doll I and Lily II over those from Flora II and Clara. In the higher doses there is an advantage for the former, but in lower doses the reverse is the case. In both cases, however, the differences are more or less within the limits of experimental error. The butters from the cows which received the higher doses of cod-liver oil are decidedly antirachitically superior to those from Flora II and Clara and much more so to those from the cows Gertie and Rufa, which were on a very restricted diet. This confirms our last year's results.

Two striking features emerge from these experiments. First, the fact that the administration of a daily dose of 2 oz. of cod-liver oil to a good winter stall ration made no very significant difference to the vitamin D content of the butter. Whether this is accidental or whether a dose of cod-liver oil below a certain magnitude does not influence antirachitically the milk will be decided by our future work. Second, the comparatively high antirachitic value of the basal diet received by Flora II and Clara. In comparing this diet with the very restricted diet of Gertie and Rufa, it is found that, apart from the slight variations in the composition of the concentrates, the main difference lies in the inclusion of silage and hay in the former diet. It is also to be observed that Gertie and Rufa were stalled all the time. From preliminary results at our disposal we feel disposed to favour the view that the silage was responsible for the vitamin D potency of the ration. In this case too we hope to obtain further experimental evidence.

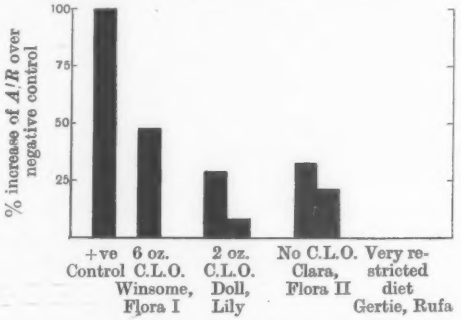


Fig. 2.

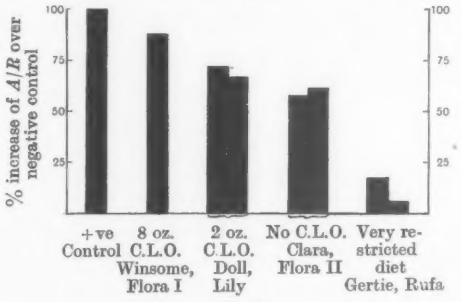


Fig. 3.

Fig. 2 represents results with 0·2 g. of butter, Fig. 3 with 0·6 g. of butter. In both figs. the ordinates represent the percentages of the increase of $A/R$ ratio above the figures of the negative control. Increase of positive control over negative control = 100.

The probable antirachitic value of silage raises an interesting problem from a theoretical as well as from a practical point of view, and is illustrative of the general importance of investigating dairy products from the vitamin

standpoint. It is particularly necessary to stress this at the present juncture of vitamin research. The dramatic discovery of the activation of foodstuffs by irradiation opened up a field of fascinating problems for research, mainly of a fundamental nature. The possible bearing of these problems on practical dietetics is, of course, patent. Does, however, our present scientific, and above all clinical, knowledge of the subject justify the cautious scientific investigator in urging the immediate application of this very incomplete knowledge to practical nutrition? The position with respect to active natural products is different. The antirachitic value of cod-liver oil and, to a smaller extent, of milk and butter, has been established clinically almost from time immemorial. The development of vitamin research has only offered a glimpse into their mode of action, thus affording a means of studying the best conditions for their production and utilisation. There is a great possibility that substances activated by irradiation may find an important place in therapeutics, but, as the ideal aim in dietetics must obviously be in the main prophylactic, the vitamin value of natural products in nutritional hygiene should not be even temporarily eclipsed by the consequences of the uncontrolled enthusiasm of the laboratory worker.

SUMMARY.

A winter ration for cows containing silage and hay is described, which produced an antirachitic butter of moderate potency. The daily addition of 2 oz. of cod-liver oil to this ration did not significantly depress the percentage of milk-fat nor did it raise the vitamin D of the butter to any appreciable extent. Higher doses of cod-liver oil depressed the percentage of milk-fat and raised the antirachitic potency of the butter.

Our thanks are due to Mr James Mackintosh who arranged for the feeding and management of the cows and to Miss Janet Robison who sampled the milk and weighed the foods for the cows.

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Drummond, Channon, Coward, Golding, Mackintosh and Zilva (1924). *J. Agric. Sci.* **14**, 531.
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# XXVI. THE ESTIMATION OF HIPPURIC AND PHENACETURIC ACIDS IN URINE.

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Hippuric acid formation, as an example of a simple synthetic process occurring in the body, has long excited interest, and methods for the isolation and estimation of this acid are very numerous. In the course of an investigation on the metabolism of phenyl-fatty acids, existing methods were reviewed and a comparison of the reliability of some of them was attempted.

The methods of estimation fall into three main classes. In the earlier the hippuric acid was extracted from the urine by some solvent and subsequently purified with as little loss as possible. In a few cases it was decomposed, after extraction, by boiling with either acid or alkali and the benzoic acid so produced isolated and weighed. The best of these methods and probably the only accurate one, that of Dakin [1910], is much too long to be of use where a number of analyses have to be performed.

In the second type of method the glycine from the extracted hippuric acid, after hydrolysis, is estimated by a formaldehyde titration [Henriques and Sørensen, 1909; Quick, 1926] or by a Kjeldahl estimation [Snapper and Laqueur, 1923]. These methods differentiate hippuric acid from benzoic acid and benzoylglycuronic acid, both of which may occur in urine, and are only to be recommended where error would be introduced by mistaking benzoylglycuronic acid for hippuric acid.

In the third type of method, hippuric acid is decomposed before extraction by boiling the urine with alkali or acid, the benzoic acid so produced being extracted and either weighed or titrated. The most rapid method is Kingsbury and Swanson's modification [1921] of Folin and Flanders's original method [1912], in which a washed chloroform extract is titrated with standard sodium ethoxide. This method was found to give good results with urines containing a not too small amount of hippuric acid but in the case of normal dog's urine, which contains on an average only 0·08 g. of hippuric acid *per diem* (equivalent to 4·5 cc. 0·1 *N* sodium ethoxide), it was found that the chloroform extract was much too yellow to enable a titration to be performed with any degree of accuracy. The method of Hryntschak [1912] in which the urinary pigments are oxidised by means of potassium permanganate prior to ether extraction was found to yield pure samples of benzoic acid and to be moderately accurate. It cannot, however, be considered to give quantitative

results when the amount of benzoic acid present is only small, in view of the observations of Kingsbury and Swanson on the effect of potassium permanganate in reducing the titration value under such circumstances. In the method of Steenbock [1912] which was worked out on cow's urine, the urine, after boiling with caustic soda to hydrolyse the hippuric acid with the addition of hydrogen peroxide to oxidise pigments, is rendered acid and extracted with ether. The benzoic acid in the residue after evaporation of the ether is sublimed out in a suitable apparatus, collected and weighed. This method was found, at first, to give not very satisfactory results, the quantitative sublimation of benzoic acid in particular affording much difficulty. As, however, it was clear that this method offered certain advantages over all others, especially for the investigation mentioned above, various modifications were introduced until ultimately a satisfactory technique was evolved. The advantages are twofold.

In the first place, if hippuric acid and phenaceturic acid are present together in urine, an investigation of their properties as well as of the properties of benzoic and phenylacetic acids and their salts showed that there was too much similarity to hope for any quantitative separation based on relative solubilities. The suggestion of Steenbock that, in his method, a titration of the sublimate with standard alkali gives a clue to the relative amounts of benzoic and phenylacetic acids present seemed to indicate the most satisfactory lines on which to work. The second advantage of this method is its applicability to the estimation of the small amounts of hippuric acid normally found in dog's urine. As already mentioned it is just under these circumstances that other possible methods become unreliable.

The method as described by Steenbock was modified in only one respect in the part prior to sublimation. The benzoic or phenylacetic acid is extracted from a solution fairly strongly acidified with sulphuric acid and a certain amount of the aqueous layer is inevitably carried over with the ether. In this way traces of sulphuric acid may be introduced into the sublimation tube and attack the benzoic acid on heating. To avoid this the ethereal solution was washed with 10–15 cc. of Folin-Flanders salt solution (saturated sodium chloride with 0·5 cc. concentrated hydrochloric acid to a litre).

The efficient sublimation of benzoic and phenylacetic acids offered most difficulty. Steenbock recommends a condensing tube 25 cm. long and 9 mm. bore with bulbs 3 cm. in diameter blown at 9 mm. intervals and filled with glass wool. If the benzoic acid was merely to be weighed this method served admirably, but where it was desired to titrate the sublimed acid it was found very difficult to wash it out completely from the glass wool and if the latter was removed from the apparatus and suspended in the solution during the titration low results were obtained owing to its slight alkalinity. Glass wool after washing loses its power as a filter. Cotton wool was therefore adopted as a filter and was found to be more efficient than glass wool in every respect. A thickness of 5–7 cm. is quite sufficient to retain the acids, whereas, a

column of at least 20 cm. of glass wool is required. Cotton wool as bought was found to be slightly acid. It was, therefore, washed several times with distilled water followed by 90 % alcohol just neutral to phenolphthalein and dried, when 1·25 g. (the approximate amount used in an experiment) suspended in neutral alcohol did not require more than 0·20 cc. *N*/20 caustic soda to render the alcohol and wool distinctly alkaline to phenolphthalein. Specimens from each batch of prepared wool were tested and a constant of 0·10 to 0·20 cc. deducted from the burette reading according to the result.

The method as ultimately adopted is as follows. 50 to 100 cc. of urine, concentrated to the latter volume if necessary, are taken and sufficient sodium hydroxide is added to make a 20 % solution of this reagent. The mixture is boiled for from 3 to 4 hours in a 500 cc. Kjeldahl flask with a condenser over a low flame. During this period up to 30 cc. of 20 vols. hydrogen peroxide are added in portions if the urine is concentrated or darkly coloured. This reagent must be avoided if it is desired to search for higher homologues of phenylacetic acid in the sublimate. The contents of the flask are then cooled and 50 % sulphuric acid added down the condenser till the mixture is faintly acid to litmus. Bromine water may now be added to precipitate phenolic bodies if these are present. The solution is filtered through

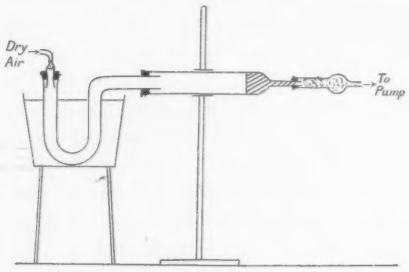


Fig. 1.

a Büchner funnel, the flask and funnel being carefully washed with three or four separate quantities of water. Sulphuric acid (50 %) is now added to render the solution strongly acid and the solution shaken out five times with ether using 150 cc. in all. The ethereal solution is transferred to a separating funnel, washed with 10 to 15 cc. of the saturated salt solution already described and then run in portions into a U-tube immersed in water at 50° through which a current of air is drawn. When all the ether is evaporated the U-tube is evacuated whilst still in the water-bath, when any water present passes off. It is now attached to a sublimation tube and the acids present sublimed.

The apparatus is represented in Fig. 1. The U-tube of diameter 2·5 cm. is immersed in a bath of molten paraffin wax and the temperature maintained at 130°. The sublimation tube for the reception of the sublimed acids is 23 cm. long and 3 cm. in diameter, and is drawn out at one end to

a diameter of 0·5 cm. so as to pass through a rubber bung into a guard tube filled with glass wool. This is weighed before and after the experiment and serves to make sure that the whole of the acids have been stopped by the cotton wool. A layer of cotton wool about 5 cm. in length is placed at the end of the sublimation tube so that half the length is in the wider and half in the narrower part. The U-tube passes into the sublimation tube through a narrow band of rubber, cut out from a rubber bung. Air, dried by passage through a sulphuric acid wash-bottle, is drawn through the apparatus by a filter pump at not too rapid a rate. Any acid condensing on the horizontal part of the U-tube is driven over from time to time by a small flame. The sublimation tube (with cotton wool) is allowed to stand overnight in a desiccator containing calcium chloride prior to the experiment and during the experiment is weighed at intervals till constancy is attained. It is again allowed to stand overnight in the desiccator before the final weighing. The length of time required for efficient sublimation is from 4 to 6 hours according to the amount of foreign material mixed with the crude acids. After the final weighing, the benzoic or phenylacetic acid, or mixture of the two, is transferred to a wide-mouthed flask along with the cotton wool and neutral 90 % alcohol added. The sublimation tube is thoroughly washed with alcohol, the solution added to that in the flask and the whole titrated with $N/20$ caustic soda free from carbon dioxide with efficient stirring. This serves as a check on the weight if the pure acids are present alone and, with mixtures of benzoic and phenylacetic acid, it serves to determine the relative amount of each. To calculate this the weight of sublimate corresponding to the titration if all were benzoic acid and if all were phenylacetic acid is found. The actual weight of sublimate lies between these figures and the proportion of either acid can be found by the simple mixture rule. In a similar way higher sublimable homologues of phenylacetic acid ($\beta$-phenylpropionic acid sublimes readily under the above conditions) or sublimable hydroxy- and keto-acids when present mixed with phenylacetic acid can be detected.

If it is desired to estimate free benzoic or phenylacetic acid as distinct from their conjugated forms the method described in Steenbock's original paper may be employed.

Duplicate experiments on different samples of urine by this method giving 0·2 to 0·3 g. of sublimate were found to agree to within 0·007 g. Using a dog's urine of known hippuric acid content an average of 97 % of added hippuric acid was recoverable as benzoic acid. The accuracy is, therefore, as great as is claimed for other methods.

With a weight of sublimate of 0·2 g., an error of 0·1 cc. $N/20$ caustic soda represents, when calculating the relative amounts of benzoic and phenylacetic acids present, a 3 % error. As an error of 0·25 cc. was found to be possible in the titration an accuracy of more than 7·5 % in this figure cannot be claimed.

SUMMARY.

(1) A method for the accurate estimation of the total benzoic acid normally present in urine is described.

(2) By means of this method the relative amounts of phenylacetic and benzoic acids, when occurring together in urine, can be estimated.

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# XXVII. A QUANTITATIVE STUDY OF THE OXIDATION OF PHENYL-FATTY ACIDS IN THE ANIMAL ORGANISM.

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The theory that fatty acids undergo in the body a series of successive oxidations at the $\beta$-carbon atom rests upon several pieces of evidence. Knoop [1904] first came to this conclusion as the result of experiments which depend on the fact that benzoic and phenylacetic acids in the body are resistant to oxidation. The administration of $\beta$-phenylpropionic acid or of $\delta$-phenylvaleric acid to a dog led to the appearance of benzoic acid (conjugated with glycine) in the urine, whereas $\gamma$-phenylbutyric acid under similar circumstances yielded phenylacetic acid in its conjugated form. It thus appears that two carbon atoms at a time are removed until one of the resistant acids is produced and excreted. The theory received further support from the observations of Embden and his co-workers [1906, 1908], on the perfusion of the surviving liver with blood containing salts of fatty acids. It was shown that only those acids containing an even number of carbon atoms show an increase in acetone formation. Any objection on the grounds of the absence of an *in vitro* analogy was removed by the discovery of Dakin [1908, 1, 2, 3], that the action of hydrogen peroxide on the ammonium salts of fatty acids leads to the formation of $\beta$-oxidation products.

More recently, however, it has been shown that with this reagent types of oxidation other than $\beta$-oxidation may occur. The action of hydrogen peroxide on the ammonium salts of a number of straight chain acids of the fatty series was found by Clutterbuck and Raper [1925] to yield products proving the existence of both $\gamma$- and $\delta$- as well as of the more usual $\beta$-oxidation. It was suggested that $\gamma$- and $\delta$-oxidation might occur in the body and be followed by rupture of the chain between the $\gamma$- and $\delta$-carbon atoms thus splitting off succinic acid which is known to be readily oxidised *in vivo*. This scheme would result in the splitting off of four carbon atoms at a time and hence lead to the same end product as the $\beta$-theory. In this way a theory of $\gamma$- or $\delta$-oxidation would explain Embden's results already mentioned and the physiological oxidation of $\delta$-phenylvaleric acid as well as the accepted $\beta$-oxidation theory. It thus seemed possible that in the case of this acid $\beta$-, $\gamma$- and $\delta$-oxidation all occurred and gave rise to the same product. With $\gamma$-phenylbutyric acid, on

the other hand, $\gamma$-oxidation might possibly give rise to some benzoic acid which would be excreted along with the phenylacetic acid obtained by $\beta$-oxidation. In view of this possibility, it seemed worth while to repeat the experiments of Knoop [1904] and Dakin [1908, 5] on this acid to see whether after its administration any benzoic acid above that normally present could be detected in the urine.

The series of phenyl-fatty acids administered by Knoop [1904] and Dakin [1908, 4, 5; 1909, 1] to dogs give as their end products either benzoic or phenylacetic acid. Now benzoic acid when fed to man can be recovered almost quantitatively (96 %) from the urine [Dakin, 1909, 2]. When fed to or injected into dogs, Delprat and Whipple [1921] found that from 70 to 100 %, according to the particular dog used, was excreted in the urine in the form of hippuric acid. If these acids are the sole end products of the oxidation of the higher acids of the same series, then for each molecule of the higher acid, one molecule of benzoic or phenylacetic acid, as the case may be, should be produced. When Dakin's figures are converted so as to represent the quantity of hippuric or phenaceturic acid recovered compared with that theoretically possible if simple quantitative $\beta$-oxidation and complete excretion occurred, we get recoveries of 41 to 53 % (in different experiments) for $\beta$-phenylpropionic acid and 28 to 33 % for $\gamma$-phenylbutyric acid. Dakin gives no figure for $\delta$-phenylvaleric acid but Knoop who does not mention the yield with the above two substances obtained a 33 % recovery in this case. It must be remembered, when comparing these results with those obtained by us, that, on the one hand, the urine was not worked up in such a way as to obtain the whole of the excreted acids and, on the other, the doses given were relatively large, from 0·5 to 1·0 g. per kg. However, it seemed that the 40 to 70 % of these acids which remained unaccounted for might easily have been converted in part into a $\gamma$-oxidation product or suffered some other change in the organism and been lost during the working up. It was therefore decided to administer to a dog as many normal phenyl-fatty acids as could conveniently be synthesised in order to see to what extent the products could be recovered when definitely quantitative methods were employed.

Some difficulty was at first found in devising a suitable method for the estimation of a mixture of benzoic and phenylacetic acids but one was eventually evolved and is described in a separate paper [Wayne, 1928]. It was realised that account would have to be taken of the amount of hippuric acid normally present in dog's urine and this was estimated before and after each experiment and allowed for. The amounts injected were such as to yield, if completely $\beta$-oxidised, approximately 1·0 g. of benzoic or phenylacetic acid, *i.e.* with the dogs employed a dose of only 0·10 to 0·20 g. per kg.

A list of the acids administered together with the results obtained is given in Table I. It will be noticed that, in the case of benzoic and phenylacetic acids, recoveries of approximately 80 % were obtained in the case of dogs "A" and "C" and 85–90 % in the case of dog "B."

Table I.

| Dog | Acid administered | | % recovery | Excreted (conjugated with glycine) as |
|---|---|---|---|---|
| A | Benzoic acid | | 78 | Benzoic acid |
| A | Phenylacetic acid | (i) | 78 | Phenylacetic acid |
| | | (ii) | 79 | |
| A | $\beta$-Phenylpropionic acid | | 77 | Benzoic acid |
| A | $\gamma$-Phenylbutyric acid | | 80 | Phenylacetic acid |
| A | $\delta$-Phenylvaleric acid | (i) | 74 | Benzoic acid |
| | | (ii) | 75 | |
| B | Benzoic acid | | 85 | Benzoic acid |
| B | Phenylacetic acid | | 90 | Phenylacetic acid |
| | $\epsilon$-Phenylcaproic acid | | 88 | ,, |
| | $\iota$-Phenyldecoic acid | (i) | 64 | ,, |
| | | (ii) | 60 | |
| C | Benzoic acid | | 79 | Benzoic acid |
| C | $\theta$-Phenylnonoic acid | | 64 | ,, |

Delprat and Whipple [1921] were unable to correlate a similarly varying incomplete recovery of benzoic acid with age, sex, dose or any other factor. The findings of Quick [1926] agree in this. For the present purpose, however, it is only necessary to compare the figures for these two acids with those for the remainder where oxidation of the side chain has occurred in the organism. It will be seen that, in the case of the acids containing 3, 4, 5 and 6 carbon atoms in the side chain the figures are identical within experimental error with the benzoic or phenylacetic acid figures. Moreover by titration it was possible to show that the final acid obtained was always that demanded by the $\beta$-oxidation theory nor were any higher homologues detected. This is of especial interest in the case of $\gamma$-phenylbutyric acid where $\gamma$-oxidation might have been expected to give rise to some benzoic acid along with the phenylacetic acid produced by $\beta$-oxidation. It has therefore been demonstrated that complete quantitative $\beta$-oxidation occurs with the four simplest phenyl-fatty acids capable of undergoing this process.

With the two longer chain acids containing 9 and 10 carbon atoms in the side chain definitely lower figures were obtained than would be expected if such quantitative $\beta$-oxidation had occurred. The fate of the equivalent of the 25 to 30 % of phenylacetic acid unaccounted for in the case of $\iota$-phenyldecoic acid as of the equivalent of the 15 % of benzoic acid in the case of $\theta$-phenylnonoic acid is not easily explained. There are some phenyl derivatives which are completely oxidised to carbon dioxide and water in the animal organism. These are notably the $\alpha$-amino- and $\alpha$-keto-phenylpropionic acids and some of their nuclear substitution derivatives, and it may be that a proportion of these higher acids is converted to some such completely oxidisable product and so escapes detection. It is also conceivable that the higher acids take longer to oxidise than the lower acids because the greater length of the side chain would necessitate a greater number of successive $\beta$-oxidations. It may happen therefore, owing to this greater length of time during which the acids with the longer side chains are undergoing oxidation, that a greater opportunity for oxidation and disruption of the benzene nucleus will occur and this would result in a smaller yield of benzoic or phenylacetic acid as the

case may be. Whatever the explanation, quantitative $\beta$-oxidation still remains unproved in the case of these long chain phenyl-fatty acids and it must be emphasised that they correspond more closely to the fatty acids normally undergoing metabolism in the body than do the lower members of the series.

It was possible to show that the final acid excreted as a result of the oxidation of these two acids also was solely that required on the hypothesis of the removal of two or a multiple of two carbon atoms at a time. There was, moreover, no indication that the oxidation of the acid administered was not completed within the experimental period. The amount of benzoic or phenylacetic acid excreted in the after period was not different from that obtained in other experiments with the simpler acids. Moreover, no acid intermediate between the acid administered and benzoic or phenylacetic acid in the two cases could be detected. The demonstration by Dakin [1909, 1] of derivatives of phenylpropionic acid in the urine after the administration of $\delta$-phenylvaleric acid occurred after large doses (0·75 g. per kg.) of the latter acid had been given.

Flaschenträger and co-workers in a series of papers [see Flaschenträger 1926] have examined the conduct in the body of a series of substituted $\omega$-amino-fatty acids of general formula $C_6H_5SO_2N(CH_3).(CH_2)_n.COOH$. These undergo $\beta$-oxidation in a similar fashion to the phenyl-fatty acids and are excreted as the unoxidisable propionic or butyric acid of the series according to the number of carbon atoms in the fatty acid chain. The results of these workers though not strictly quantitative are very similar to those obtained by us. Quantitative $\beta$-oxidation seems to occur with the lower members of this series and no intermediate products were detected even with the higher members.

The question of the nature of the intermediate products formed during the oxidation of even so simple a substance as $\beta$-phenylpropionic acid is by no means settled. It is unlikely that a single reaction is involved in the shortening of the carbon chain by two carbon atoms in the course of a $\beta$-oxidation and it has been suggested that oxidation of a saturated acid gives rise, in the first stage, to the $\alpha$-$\beta$ unsaturated acid, *i.e.* cinnamic acid in the above case rather than to a $\beta$-keto- or $\beta$-hydroxy-acid.

Kay and Raper [1924] found when dogs were injected with $\beta$-phenyl-$\alpha$-methylpropionic acid and $\alpha$-methylcinnamic acid respectively that whereas the former yielded 77 % of the expected amount of benzoic acid if all had undergone $\beta$-oxidation the latter under comparable conditions only gave 30 %. It was concluded, therefore, that the formation of the unsaturated acid was not an obligatory stage in the oxidation of the saturated acid, for otherwise the yields should have been about the same. It seemed possible that conjugated double bonds might in general confer, as in this case, a greater susceptibility to oxidation upon the benzene nucleus. It was hoped that an examination of the extent to which the oxidation products of cinnamic and phenyl-*iso*-crotonic acids could be recovered when compared with the results in the case of $\beta$-phenylpropionic and $\gamma$-phenylbutyric acids might throw similar light

on the problem of the mode of oxidation of these latter substances. The results are given in Table II. It will be seen that these unsaturated acids yield β-oxidation products to the same extent, allowing for experimental error, as the saturated acids and therefore remain quite possible intermediate substances in the course of oxidation of the latter.

Table II.

| Dog | Acid administered | % recovery | Excreted (conjugated with glycine) as |
|---|---|---|---|
| A | β-Phenylpropionic acid | 77 | Benzoic acid |
| A | Cinnamic acid | 74 | ,, |
| A | γ-Phenylbutyric acid | 80 | Phenylacetic acid |
| A | Phenyl-*iso*-crotonic acid | 77 | ,, |

## Experimental.

### *Preparation of materials.*

*γ-Phenylbutyric acid.* The most rapid method of preparing this acid is from γ-phenyl-*n*-propyl alcohol. This was converted to γ-phenyl-*n*-propyl bromide by the method of Rupe and Bürgin [1910]. The compound with magnesium was then formed by the Grignard reaction and this was decomposed by carbon dioxide giving the required acid [Rupe and Proske, 1910]. In this way 8 g. of pure acid were obtained from 27 g. of the alcohol.

*δ-Phenylvaleric acid.* Sodium (3·3 g.) was dissolved in alcohol (50 cc.) and to the well-cooled solution diethyl malonate (23 g.) was added followed by γ-phenyl-*n*-propyl bromide (28 g.). After standing for half an hour the mixture was heated on the water-bath for 3 hours, cooled, water added and the oil extracted with ether. The ether was distilled off after drying and the oil fractionated *in vacuo*. The diethyl δ-phenyl-*n*-propylmalonate (24 g.) so obtained was collected at 190–200°/15 mm. and hydrolysed by boiling with alcoholic potash for 2 hours. The alcohol was evaporated and the residue dissolved in water was treated with hydrochloric acid precipitating δ-phenyl-*n*-propylmalonic acid. This agreed in its properties with the acid differently prepared by Borsche [1912]. The acid was heated *in vacuo* till no further gas was evolved and then distilled *in vacuo* when δ-phenylvaleric acid (6 g.) passed over at 190–193°/30 mm. and was recrystallised from hot water, when it had M.P. 58·5–59·5°.

The higher acids were prepared by the same general method. The appropriate dibasic acid of the succinic series was converted to its acid ethyl ester and thence to the carbethoxy-acyl chloride. By the Friedel-Crafts reaction with benzene this gave an ω-benzoyl-fatty acid ester which was reduced to the required phenyl-fatty acid by Clemmensen's method [1914].

*ε-Phenylcaproic acid.* Adipic acid was converted to ethyl hydrogen adipate by esterification with insufficient alcohol as described by Blaise and Koehler [1910]. The normal ester also produced in this reaction was then semi-hydrolysed by the method of Grün and Wirth [1922] described for the semi-hydrolysis of ethyl sebacate. Ethyl hydrogen adipate was converted to the

acid chloride by means of thionyl chloride. δ-Carbethoxyvaleryl chloride (25 g.) in dry benzene (200 g.) was treated with anhydrous aluminium chloride (33 g.). After the vigorous initial reaction had subsided the mixture was heated on the water-bath for an hour when it was cooled and poured on to ice with the addition of hydrochloric acid. From the benzene layer, washed with water and sodium bicarbonate solution and dried, the benzene was distilled off and the residue vacuum-distilled. In this way ethyl δ-benzoylvalerate (16 g.) was obtained and collected at 200–210°/30 mm. A little on hydrolysis gave δ-benzoylvaleric acid agreeing in properties with that described by Bauer [1914]. The ester (15 g.) was placed in a flask with amalgamated zinc [Clemmensen, 1914], alcohol (100 cc.) and 30 % hydrochloric acid (100 cc.) and the whole boiled vigorously for 20 hours during which time an additional 300 cc. of 30 % hydrochloric acid was added. The cooled contents were then extracted with ether, the ether evaporated and any ester remaining was hydrolysed by boiling with caustic soda for half an hour. The acid liberated by treatment with hydrochloric acid was extracted with ether and distilled *in vacuo*. The fraction collected at 190–205°/25 mm. gave a low equivalent. It was dissolved in caustic soda, the solution extracted with ether and after acidification the acid extracted with light petroleum. In this way ε-phenylcaproic acid was obtained after evaporation of the light petroleum, equivalent 191·5 (calculated 192). It is a liquid agreeing in its properties with that described by v. Braun [1911].

*θ-Phenylnonoic acid.* Azelaic acid was converted to ethyl hydrogen azelate by the same methods as were used for adipic acid. The acid ester was converted to η-carbethoxy-*n*-octoyl chloride by means of thionyl chloride [Robinson and Robinson, 1926]. The Friedel-Crafts reaction was conducted precisely as is described above for the lower homologue. On evaporating the benzene at the end of the reaction a mixture of crude acid and crude ester was obtained in this case. This was treated with alcohol saturated with hydrogen chloride so as to convert the whole to ester and the ester distilled *in vacuo*, the fraction boiling between 175–200°/12 mm. being collected and hydrolysed. The η-benzoyloctoic acid so obtained was recrystallised from a large amount of light petroleum to separate it from traces of azelaic acid and finally recrystallised from dilute alcohol. The acid forms fine colourless irregular plates from this solvent; M.P. 74·5–75°. Equivalent 249·5 (calculated 248).

Analysis of η-benzoyloctoic acid: found, C 72·67, H 8·18 %; calculated for $C_{15}H_{20}O_3$, C 72·59, H 8·64 %.

The acid (0·4 g.) heated with semicarbazide hydrochloride (0·26 g.) and sodium acetate (0·5 g.) in 90 % acetic acid for half an hour on the water-bath gave the semicarbazone of η-benzoylnonoic acid. This separates from alcohol in fine needles; M.P. 151·5–152°.

Analysis: found, C 62·5, H 7·3 %; calculated for $C_{16}H_{23}O_3N_3$, C 62·9, H 7·5 %.

The acid was esterified and reduced by Clemmensen's method in the same way as is described above for δ-benzoylvaleric acid. θ-Phenylnonoic acid so produced distils at 215°/12 mm. and solidifies in ice to a waxy solid; M.P. 29–30°. Equivalent 233 (calc. 234).

Analysis: found, C 77·03, H 9·08 %; calculated for $C_{15}H_{22}O_2$, C 76·93, H 9·39 %.

*ι-Phenyldecoic acid.* θ-Carbethoxy-*n*-nonoyl chloride was prepared from sebacic acid by the method described above for the valeryl and octoyl compounds. By the Friedel-Crafts reaction conducted as has been described for these latter compounds this was converted into θ-benzoylnonoic acid, M.P. 84°, identical in properties with that described by Borsche and Wollemann [1911] by whose method it was also made but with less satisfactory results. The ethyl ester of θ-benzoylnonoic acid was reduced by Clemmensen's method to ι-phenyldecoic acid and purified as described in the case of ε-phenylcaproic acid. ι-Phenyldecoic acid, distilling at 196°/0·4 mm., is a white waxy solid; M.P. 42°. Equivalent 249 (calc. 248). It has been described by Borsche [1919] and the specimen agreed in its properties with the description of this observer.

### General method of conducting the experiments.

The subjects of the experiments, fox terrier bitches of age 1–2 years and about 10 kg. weight, were fed on a standard diet consisting of Spratt's ovals, potatoes and lard. The amounts were varied until the animal maintained a constant weight. During an experiment the animal was kept in a metabolism cage. Each morning it was transferred to a fresh cage and the urine passed during the preceding 24 hours was collected, the cage being well washed down with about a litre of water. The urine and washings were filtered, made slightly alkaline and boiled down to a small bulk on the water-bath.

For two days before an injection the urine was collected and the benzoic acid present during this time determined to give the *initial blank*. The acid to be injected was weighed out, carefully neutralised with standard sodium hydroxide and made up to about 25 cc., the solution being weighed. In the case of cinnamic acid the ammonium salt was employed, as sodium cinnamate is rather insoluble in water. About 10 cc. of solution were taken up from a known weight into a hypodermic syringe and injected into the dog, the site of injection being the loose subcutaneous tissues of the posterior cervical region. The weight of solution injected was determined by difference. The appropriate amount of solution in the case of phenylnonoic and phenyldecoic acids was divided into two and injected on two consecutive days, as these acids tended to produce toxic symptoms in the animal. The urine was collected for 3 days following the last injection, the specimens added together, boiled down to a small bulk and made up to 250 cc. Duplicate determinations were then made on measured volumes of the solution giving the *experimental figure*.

Table III.

| Dog | Acid administered | Amount injected g. | Equivalent to benzoic (B) or phenylacetic (P) acid g. | Initial blank benzoic acid g. *per diem* | Final blank benzoic acid g. *per diem* | Average blank benzoic acid g. *per diem* | Amount of benzoic acid normally in urine during experimental period (*Y*) g. | Total of acids excreted during experimental period (*X*) g. | Average of estimations of (*X*) g. | Excess of acid present during experimental period *X*−*Y* g. | Recovery % |
|---|---|---|---|---|---|---|---|---|---|---|---|
| A 10·5 kg. | Benzoic | 1·00 | 1·00 (B) | 0·040 | 0·058 | 0·049 | 0·147 | 0·910<br>0·940 | 0·925 | 0·778 | 78 |
| A | Phenylacetic | 1·02 | 1·02 (P) | 0·045 | 0·063 | 0·054 | 0·162 | 0·957<br>0·953 | 0·955 | 0·793 | 78 |
| A | ,, | 1·045 | 1·045 (P) | 0·063 | 0·035 | 0·049 | 0·147 | 0·973 | 0·973 | 0·826 | 79 |
| A | Phenylpropionic | 1·28 | 1·04 (B) | 0·031 | 0·064 | 0·047 | 0·141 | 0·935<br>0·945 | 0·940 | 0·799 | 77 |
| A | Phenylbutyric | 1·23 | 1·025 (P) | 0·050 | 0·040 | 0·045 | 0·135 | 0·945<br>0·975 | 0·960 | 0·825 | 80 |
| A | Phenylvaleric | 1·42 | 0·975 (B) | 0·037 | 0·057 | 0·047 | 0·141 | 0·855<br>0·895 | 0·875 | 0·734 | 75 |
| A | ,, | 1·45 | 0·990 (B) | 0·038 | 0·071 | 0·054 | 0·162 | 0·895<br>0·900 | 0·897 | 0·735 | 74 |
| A | Cinnamic | 1·20 | 0·990 (B) | 0·032 | 0·062 | 0·046 | 0·138 | 0·873<br>0·860 | 0·866 | 0·728 | 74 |
| A | Phenyl-*iso*-crotonic | 1·17 | 0·980 (B) | 0·055 | 0·055 | 0·055 | 0·165 | 0·917 | 0·917 | 0·752 | 77 |
| B 8·7 kg. | Benzoic | 1·03 | 1·03 (B) | 0·069 | 0·066 | 0·067 | 0·201 | 1·080 | 1·080 | 0·879 | 85 |
| B | Phenylacetic | 1·04 | 1·04 (P) | 0·067 | 0·049 | 0·058 | 0·174 | 1·112 | 1·112 | 0·938 | 90 |
| B | Phenylcaproic | 1·37 | 0·975 (P) | 0·057 | 0·064 | 0·060 | 0·180 | 1·045<br>1·040 | 1·042 | 0·862 | 88 |
| B | Phenyldecoic | 1·79 | 0·985 (P) | 0·052 | 0·053 | 0·0525 | 0·157 | 0·777<br>0·800 | 0·789 | 0·632 | 64 |
| B | ,, | 1·81 | 0·995 (P) | 0·042 | 0·032 | 0·037 | 0·148 (4 days) | 0·735<br>0·744 | 0·740 | 0·592 | 60 |
| C 7·0 kg. | Benzoic | 1·01 | 1·01 (B) | 0·034 | 0·039 | 0·0365 | 0·108 | 0·900<br>0·910 | 0·905 | 0·797 | 79 |
| C | Phenylnonoic | 1·945 | 1·01 (B) | 0·040 | 0·036 | 0·038 | 0·152 (4 days) | 0·795<br>0·800 | 0·797 | 0·645 | 64 |

The amount of benzoic acid present in the urine during 2 days following the experimental period was also estimated giving the *final blank*. This served to ensure that the benzoic acid excretion had fallen back to its normal value. In agreement with the observations of other investigators it was found that 3 days were sufficient for the complete excretion of products of injected acids of this type.

The average of the *initial* and *final blanks* was used to determine the amount of benzoic acid which would normally have been excreted during the experimental period and this was deducted from the *experimental figure*.

The recovery was then expressed as the percentage of that which would have occurred if one molecule of the acid injected had given one molecule of phenylacetic or benzoic acid, as the case may be, and the whole of this had been excreted.

The experimental figures are given in Table III. It will be seen that in the different dogs the blank figures are fairly constant, especially the average blank which is the average over 4 days. Some error is, however, necessarily introduced in calculating the amount of benzoic acid normally present in the urine during the experimental period from this figure. There is a further possible source of error in the estimation of the acid excreted during the experimental period and it is probable that differences of less than 5 % in the figure expressing the recovery are due to experimental error. In those cases where phenylacetic acid was the end product of oxidation the benzoic acid excreted along with it as the result of the normal metabolism of the dog was estimated by titration of the weighed sublimate. This figure for benzoic acid agreed within experimental error with the amount calculated from the average blank.

## Summary.

(1) Normal phenylpropionic, phenylbutyric, phenylvaleric and phenylcaproic acids when administered to dogs yield amounts of benzoic and phenylacetic acid, as the case may be, which indicate that the fatty acid side chain is oxidised quantitatively in accordance with the theory of $\beta$-oxidation.

(2) Under the same conditions, phenylnonoic and phenyldecoic acids yield smaller amounts of benzoic and phenylacetic acid respectively than would be expected if quantitative $\beta$-oxidation of the side chain takes place.

(3) This suggests that some other mode of oxidation, in addition to $\beta$-oxidation, takes place with phenyl-fatty acids containing side chains approaching in length those of the higher fatty acids, but evidence as to the nature of this additional mode of oxidation has not been obtained.

(4) Cinnamic and phenyl-*iso*-crotonic acids yield the same amounts of benzoic and phenylacetic acid respectively as the corresponding saturated acids.

We wish to express our thanks to the Government Grant Committee of the Royal Society for a grant in aid of this work and to the Medical Research Council for a grant which enabled one of us (E. J. W.) to participate in it.

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# XXVIII. STUDIES IN PURINE METABOLISM.

## V. THE NUCLEAR-PLASMIC RATIO OF FROGS.

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(*Received December 12th, 1927.*)

### Introduction.

In Parts I and IV of this series [1926, 1927] it was shown that inanition does not in the case of rats and dogs lead to an increase in the chemical nuclear-plasmic ratio, as would have been the case had protein reserves been present in the cytoplasm of these mammals. In view of this result, it was interesting to verify whether the same was applicable to poikilothermic animals such as frogs. This material is, moreover, of particular interest, for several reasons. Firstly, as shown by Przylecki and Opieńska [1926], these animals have normally an almost exclusively protein metabolism, and do not possess any considerable fat reserves. Thus, if protein reserves exist, they should above all be present in the poikilotherms. Secondly, the period over which they can endure inanition is much greater for frogs than for mammals. Thirdly, it was mainly upon the basis of the histological examination of such material that the theory of protein storage was originally put forward [Leonard, 1887; Morgulis, 1911]. Finally, since the frog undergoes certain seasonal changes, such as hibernation, sexual activity, etc., the possibility might exist that the nuclear-plasmic ratio might undergo seasonal variation.

### Experimental.

*Rana esculenta* was employed. Six or seven frogs were taken for each determination, being minced after removal of the ovaries or testes and of the contents of the alimentary tract. The experimental methods used were the same as in our previous papers, *i.e.* total nitrogen, $N_T$, was determined by the Kjeldahl method, purine nitrogen, $N_P$, by the method of Krüger and Schittenhelm, the ratio $N_P/N_T - N_P$ being taken as the chemical nuclear-plasmic ratio.

Determinations were made once or more every month from May, 1926, to June, 1927 only the average value for the month being given in the table.

Table I. *Nuclear-plasmic ratio of frogs from May, 1926, to June, 1927.*

| Month | % solid substance | % N live weight | % N dry weight | Purine-N mg. % live weight | Purine-N mg. % dry weight | Nuclear-plasmic ratio |
|---|---|---|---|---|---|---|
| May 1926 | — | 1·935 | — | 77·2 | — | $41{\cdot}55 \times 10^{-3}$ |
| July „ | 20·4 | 2·551 | 12·50 | 98·2 | 481·2 | 40·02 „ |
| Aug. „ | 20·5 | 2·700 | 13·17 | 90·6 | 442·0 | 34·83 „ |
| Sept. „ | 20·7 | 2·423 | 11·71 | 95·8 | 462·7 | 41·15 „ |
| Oct. „ | 22·3 | 2·521 | 11·30 | 92·1 | 413·0 | 37·92 „ |
| Nov. „ | 21·3 | 2·638 | 12·38 | 98·1 | 459·9 | 38·56 „ |
| Dec. „ | 20·0 | 2·585 | 12·98 | 96·8 | 483·7 | 38·82 „ |
| Jan. 1927 | 20·1 | 2·561 | 12·85 | 100·9 | 502·0 | 41·20 „ |
| Feb. „ | 18·3 | 2·657 | 14·52 | 97·0 | 530·3 | 37·91 „ |
| Mar. „ | 18·3 | 2·296 | 12·54 | 78·5 | 429·1 | 35·42 „ |
| Apr. „ | 20·7 | 3·034 | 14·62 | 89·7 | 433·4 | 30·48 „ |
| June „ | 18·5 | 2·474 | 13·38 | 85·3 | 461·2 | 35·71 „ |
| Mean | 20·1 | 2·531 | 12·81 | 91·7 | 458·2 | 37·80 „ |

The percentage dry content, at first 20·4, attains a maximum of 22·3 in October, falling to 18·3 in February, March and 18·5 in June. The total nitrogen content is lowest in May, 1926, presumably owing to high water content, sufficient material having unfortunately not been available for the determination of this value. During the rest of the year the values of $N_T$ obtained vary very little from the average of 2·531 %, the highest value being found in April (3·034 %). The total nitrogen content calculated on dry weight is lowest in September and October and highest in the spring, corresponding with the greatest and least non-protein contents respectively. The purine nitrogen content varies 10 % about the average value of 91·7 mg. %, the highest values being found during the winter months (Nov.–Feb.).

As to the nuclear-plasmic ratio, this, with one exception, is extremely close to the mean value of $37{\cdot}8 \times 10^{-3}$. Between March and June, 1927, the values obtained are lower than this average, whilst from May, 1926, to February, 1927, they are higher.

## Discussion.

In Poland, frogs do not feed between November and April, and the alimentary tract of frogs taken during this period is empty. In March or April begins the period of sexual activity, *i.e.* before feeding, and in this period considerable quantities of eggs and sperm are produced. If, therefore, specific protein reserves exist within the cytoplasm of the frog, these should be exhausted in March or April, with a corresponding rise in the nuclear-plasmic ratio. The above results show, however, that the value actually found is lower from February to April than during the preceding months. In Part I of this series [1926] it was shown that rats behave similarly in this respect, inanition also leading to diminution in the nuclear-plasmic ratio. It would appear, therefore, that amphibia as well as mammals do not lay aside any specific deposit protein.

It is of interest, further, that the purine content of frogs is about 50 % higher than that of mammals; this is possibly due to their lower body temperature (Gerassimow [1902] found that the nuclear-plasmic ratios of infusoria cultivated at 8° and at 25° were as 13 : 7).

SUMMARY.

1. The chemical nuclear-plasmic ratio of *Rana esculenta* has been determined monthly over a year.

2. The nuclear-plasmic ratio has a value of approximately double that of mammals.

3. It is highest from May to January, the value falling slightly during the periods of winter-sleep, hunger, and sexual activity which follow.

4. The existence of specific reserve protein in the cytoplasm of the frog is not confirmed.

The above research was undertaken at the suggestion of Prof. St J. Przylecki, to whom I wish to express my sincere gratitude for his constant help and advice.

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# XXIX. THE ACCURATE MICRO-DETERMINATION OF CHLORINE AND IRON IN BLOOD AND OTHER LIQUIDS.

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(*Received January 5th, 1928.*)

## Introduction.

Most micro-chemical analyses are carried out by colorimetric methods or by titration. In titration, the accuracy rests largely on the determination of an end-point. It is obvious that when the volume of fluid in which a reaction is carried out becomes doubled, at least twice the excess of standard reagent will have to be added to produce the same excess of end-point products; and if a coloured end-product has any tendency to dissociate into colourless ions, as is the case with ferric thiocyanate, then the excess required to produce an end-point of equal intensity will be more than doubled.

The success of a micro-titration method would appear, therefore, to depend on:

(1) the maintenance of reacting materials in small bulk [Rehberg, 1926];

(2) the minimisation of ionisation and reversible reactions in the end-products;

(3) the selection of a distinct colour change for the end-point.

If these factors can be controlled in small bulk, there is no reason why the error from them should be larger than for a corresponding macro-method, so that the determination will depend for accuracy on the measurement of small quantities of material, and the elimination of such errors as those due to the exposure of reagents to a greater relative surface of glassware when one is dealing with minute amounts of liquid.

When equal amounts of ferric thiocyanate are diluted to a given volume with water, alcohol and acetone, it is found that the depths of colour are in ascending order. Now ferric thiocyanate is the end-point product in the chlorine determination previously described [Smirk, 1927, 1] and the substance used for colorimetric comparison in the iron determination [Smirk, 1927, 2]. If, therefore, titration is carried out in the presence of acetone, the end-point will be sharper where ferric thiocyanate is the determining factor.

If equal quantities of ammonium thiocyanate are treated with varying amounts of ferric iron alum and diluted to a definite volume, the depth of colour increases with the amount of iron alum added, owing to the reaction

$$Fe_2(SO_4)_3 + 6NH_4CNS \rightleftharpoons 2Fe(CNS)_3 + 3(NH_4)_2SO_4$$

being displaced to the right in the presence of excess of iron alum.

As these three conditions of bulk, non-reversibility and end-point are readily maintained, the reduction of the quantity of blood, etc. used, from 0·2 cc. to 0·015 cc. or less, has been readily accomplished with only a slight loss of accuracy in the method of chloride estimation previously described. The sole difficulty was the accurate measurement of the material, which was overcome by the use of a capillary pipette which may be made and graduated with ease and without special knowledge or equipment.

In the calibration of an ordinary micro-pipette graduated to contain 0·2 cc. the error is small but greater than in ordinary macro-pipettes, because in the micro-pipettes the stem is relatively wider, so that a small linear error in reading or graduating the pipette represents a greater percentage error in the volume of the contents. The width of the stem is necessary, however, in order that the 0·2 cc. graduation mark may lie within its length. If the stem is made very narrow compared with the bulb, only a small percentage of the pipettes would have a bulb whose volume content was so accurately gauged that the calibration mark would fall on the stem.

Fig. 1. Micro-pipette. To measure accurately 1/75 cc. or less.

It will, therefore, be seen that although it may be almost a technical impossibility to make micro-pipettes to contain accurately a fixed and stated volume of liquid, such as 0·05 or 0·01 cc., yet it will be possible, by having a graduation mark over a sufficiently narrow constriction or stem, to get a pipette which measures say 0·0135 cc. very accurately indeed. The obvious difficulty in the use of such pipettes is, however, that the proportion of the essential elements in a reaction mixture such as serum and silver nitrate, tends to be disturbed and large errors result. But if the strength of silver nitrate solution is so adjusted that equal quantities of serum and silver nitrate solution give the correct silver nitrate excess in the reaction mixture, then the same pipette may be used for the addition of both liquids.

It was, therefore, decided to make a capillary pipette using as graduation mark a fine hair capillary constriction such as holds the mercury column in a clinical thermometer.

### Technical details.

A length of thick capillary tubing is drawn out so that the bore is about $\frac{3}{4}$ millimetre. It is then tapered abruptly so that the point will not readily be broken. A length of 2 or 4 cm. of this capillary tubing, containing approximately 0·02 to 0·01 cc., is almost sealed off from the main body by a micro-burner, so that a fine constriction results. The tube is allowed to cool and is cleaned with concentrated nitric acid, distilled water, alcohol, and ether; the last three being drawn through the pipette by a good suction pump.

The pipette is next filled with mercury to the middle of the capillary constriction, and this mercury is delivered into a small glass bottle and weighed to the fourth decimal place. The volume of the pipette is calculated by dividing the weight of mercury by the specific gravity of mercury at room temperature. Graduation of the pipette with mercury is extremely accurate but often, as in other micro-pipettes, tiresome owing to the difficulty of striking the middle of the arbitrary calibration mark. The best way is to fill the pipette just beyond the constriction and then to tap the tapered end of the pipette gently on a piece of filter paper held over the thumb. Tiny globules of mercury can in this way be squeezed out of the pipette and, with practice, calibration becomes easier than for the ordinary micro-pipettes of larger size, graduated to contain a definite volume of liquid.

Alternatively, the pipette has been calibrated with a strong standard solution of silver nitrate which is titrated against standard ammonium thiocyanate.

In micro-colorimetric methods the accurate measurement of small bulks of material plays a similar part, but conditions for micro-analysis are simpler, since the main factor is the dilution of the colour to the optimal concentration for colorimetric work with a diluent which minimises ionisation or reversible reactions of the coloured substances. The conditions are fulfilled in the thiocyanate method for iron determination by the use of nitric acid and excess of thiocyanate, which renders the reaction more complete, and of acetone which has the same effect by diminishing the dissociation of ferric thiocyanate.

### The accurate estimation of chlorine on 0·02 to 0·01 cc. of serum, whole blood, corpuscles, urine or sweat.

In an earlier paper [Smirk, 1927, 1] a method was described for the accurate estimation of chlorides on 0·2 cc. of blood in about 10 minutes. For combined investigations on the serum, whole blood, and corpuscular chlorides of capillary blood samples, it is necessary to work on much smaller quantities of material, since 0·2 or 0·3 cc. is all that can be obtained from a single skin puncture, without the use of procedures altering the composition of the blood, such as heating or congesting the fingers from which the samples are taken.

The rôle of the skin in chloride excretion which appears to be of great importance in nephritis, especially as regards treatment with vapour baths, has also been incompletely investigated because of the difficulty experienced in obtaining sufficient quantities of sweat for analysis. The error due to evaporation tends to be very great in the collection of the amount required for analysis by existing methods.

#### *Procedure.*

A well mixed sample of the liquid to be analysed is taken up in the pipette and accurately adjusted to the level of the constriction, soaking up the excess with pieces of thin filter paper. The contents of the pipette are delivered to

the bottom of a 9 × 1 centimetre test-tube, taking care that as much adherent fluid is removed from the outside of the pipette as possible. The pipette is next filled with distilled water to just above the graduation mark and the washings delivered $\frac{1}{2}''$ above the bottom and on one side of the test-tube, which has been marked with a glass pencil or a piece of gummed paper. This washing is repeated twice and the washings added to the contents of the test-tube with similar precautions. The distilled water should be taken from a drop hanging from a burette, so that any material from the outside of the tip of the pipette, contaminating the residue of the drop, will be delivered to the test-tube in the subsequent washings.

The pipette is next washed with distilled water, alcohol and ether and is filled to the graduation mark with silver nitrate solution. The silver nitrate is of the following strength:

For sweat and urinary chlorides 43·592 g. per litre (1 cc. = 0·015 g. NaCl).
For serum chlorides 21·796 g. per litre (1 cc. = 0·0075 g. NaCl).
For whole blood chlorides 17·437 g. per litre (1 cc. = 0·0060 g. NaCl).
For corpuscular blood chlorides 13·078 g. per litre (1 cc. = 0·0045 g. NaCl).

The silver nitrate of appropriate strength is delivered to the tube using the precaution already outlined, and the pipette is washed out four or five times with concentrated nitric acid also taken from the hanging drop of a burette.

About as much powdered ammonium persulphate as would go on a 2 mm. square is then added, and the reaction mixture heated with moderate agitation over a micro-burner until all protein is dissolved. Air is then bubbled through the liquid by a very fine capillary tube and heating is continued by the micro-burner until the silver chloride forms small discrete masses at the bottom of the tube and the solution above is clear.

The reaction mixture is then cooled and diluted with approximately an equal quantity of acetone, which is delivered down the sides of the test-tube so that it carries the particles of fluid spurts to the bottom of the test-tube. The mixture is again cooled, one large drop of saturated iron alum solution is added, and it is then ready for titration. This is carried out as described [Smirk, 1927, 1] by delivering the alcoholic ammonium thiocyanate (20 cc. = 1 cc. of $AgNO_3$ = 0·005 g. of NaCl) directly into the liquid by means of a fine capillary extension of the micro-burette, attached to it by pressure tubing of small bore, and stirring the titration mixture with a rapid stream of fine bubbles from a capillary tube.

The amount of thiocyanate added should be about 0·15 to 0·25 cc. in order that the extent of dilution should not be greatly altered. The end-point is changed distinctly by 0·003 cc. of the ammonium thiocyanate. It is, therefore, desirable that a capillary burette graduated to 0·001 cc. should be used. Excellent results can be obtained with a burette graduated in 0·01 cc. if the divisions are read with the aid of a lens, and the tenths of a division estimated. Delivery from the burette should be constant and slow, since the slightest error due to incomplete drainage will show in the results. The end-point is to

be taken at the earliest reddish flush through the solution. 0·003 cc. added after this should change the colour of the solution to a distinct red.

The accuracy of the method depends on attention to detail.

(1) Avoidance of contamination of the pipette after washing, by contact with the original contents.

(2) The very earliest perceptible change should be noted, and then the titration carried a shade beyond to confirm the observation.

(3) The use of a strong white front illumination from a shaded lamp and of a white porcelain or paper background in actual contact with the test-tube is absolutely essential for accurate titration.

(4) All the ammonium persulphate must have been destroyed by nitric acid before the acetone is added or the end-point is ruined by the oxidation of the ferric thiocyanate with consequent decoloration.

(5) The titration must be carried out in the cold. It is not wise to have a hot electric lamp close to the test-tube except during the critical phase of the titration.

### Results.

The following represent consecutive series of results on samples of serum, whole blood and corpuscles (g. NaCl per 100 cc. of serum, whole blood and corpuscles respectively).

| | *Sample A* | *Sample B* |
|---|---|---|
| Serum chloride | 0·637 | 0·627 |
| | 0·632 | 0·623 |
| | | 0·628 |
| Whole blood chloride | 0·484 | 0·492 |
| | 0·486 | 0·491 |
| | 0·490 | 0·497 |
| | | 0·485 |
| Corpuscular chloride | 0·256 | 0·327 |
| | 0·256 | 0·333 |

| | *Sample C* |
|---|---|
| Serum chloride | 0·620 |
| Corpuscular chloride | 0·327 |

Haemocrite reading: 46 % corpuscles, 54 % serum.
Whole blood chloride by calculation $= 0{\cdot}620 \times 0{\cdot}54 + 0{\cdot}327 \times 0{\cdot}46 = 0{\cdot}487$.
Whole blood chloride by estimation $= 0{\cdot}472$.

### The accurate estimation of iron in 0·02 to 0·01 cc. of whole blood or corpuscles.

The principle of this method is practically that of a scale reduction of the original method on 0·2 cc. already quoted. It is, however, more rapid and if carefully performed more accurate. This increase in accuracy is probably due to the rapidity of the process which leaves little time for alteration in the colour of the solutions to be matched.

#### *Procedure.*

Using a micro-pipette as described for the estimation of chlorides on 0·02 to 0·01 cc., a small measured quantity of blood is delivered to the bottom of a Jena glass test-tube; approximately 2 × 12 cm. The pipette is washed out

(as described p. 204) three or more times, using in all 0·1 cc. of distilled water. 0·1 cc. of concentrated nitric acid is then added.

As much ammonium persulphate as will go on a 2 mm. square is then added with care, so that all of it reaches the bottom of the Jena tube and none remains adherent to the walls above the reaction mixture. In the absence of this precaution the ferric thiocyanate colour would fade slightly during the estimation owing to the oxidising action of the ammonium persulphate. The mixture is heated gently over a micro-burner and in about 20 seconds solution of the protein is complete. The clear solution is shaken round the lower walls of the test-tube to dissolve any small particles of protein which may have escaped from the main mass.

The lower part of the test-tube is then placed in an ordinary Bunsen burner for a few seconds with gentle agitation to keep the inner glass surface of the tube covered with liquid. As soon as vapours rise half or three-quarters of the way up the test-tube, it is removed from the Bunsen flame and cooled rapidly under the tap.

The tube now contains: $x$ (say 0·01725 cc.) of blood, 0·1 cc. of water and 0·1 cc. of concentrated nitric acid.

Neglecting the slight change in volume, which will follow the addition and solution of the ammonium persulphate, the total volume is $(0{\cdot}2 + x)$ cc.

Add to the test-tube 2 cc. of distilled water from an accurate pipette and then 2 cc. of acetone, mix thoroughly and cool for 20 seconds in running water. Now add 1 cc. of concentrated ammonium thiocyanate solution (24 g. per 100 cc.) to the solution in the test-tube, mix thoroughly and cool in running water.

The ferric thiocyanate colour is compared colorimetrically with the artificial standard already described in the earlier paper.

*Calculation.* Let the unknown blood and the iron standard be set at 15 mm. in the left-hand cup of the colorimeter and let the corresponding readings of the artificial standard be "B" and "S."

Then as the dilutions are similar, $\frac{15}{B}$ cc. of blood have the same iron content as $\frac{15}{S}$ cc. of iron standard.

Therefore the iron content of the blood is $\frac{50\,B}{S}$, where 50 mg. of ferric iron per 100 cc. is the adopted strength of the standard.

*Notes and observations on the method.*

(1) Each analysis, including the cleaning of the pipette and taking of the sample, occupies about 10 minutes.

(2) The colorimeter is very carefully cleaned with distilled water, alcohol and ether before the first colorimetric comparison is made. Afterwards, the cups are merely emptied, rapidly drained and filled at once with the next of a series of solutions. This changing of solutions must be carried out rapidly, with the

minimum delay and without any attempt at washing out the cups, in order that errors due to evaporation of the water-acetone mixture may be avoided.

(3) Great care must be taken with the quantities of the washings since 0·1 cc. more or less represents a 2 % error in 5 cc. of solution.

(4) For description of the preparation of an iron standard, see Fowweather [1926].

## Results.

Consecutive estimations of whole blood iron: 48·9, 48·0, 48·7, 48·0, 47·2 mg.

These results, as will be seen from the above account of the method, were obtained by comparison with an iron standard treated in a similar fashion. There is no essential difference from the method previously described for 0·2 cc. of blood.

## Summary.

(1) Some general principles of micro-analysis have been discussed.

(2) An accurate method for the estimation of chlorine on 0·02 cc. of serum, whole blood, corpuscles, urine or sweat has been described.

(3) An accurate and rapid method for the estimation of iron on 0·02 to 0·01 cc. of whole blood or corpuscles has been described.

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# XXX. FURTHER DEVELOPMENTS OF RAPID VOLUMETRIC METHODS FOR THE DETERMINATION OF AMINO-ACIDS, ORGANIC ACIDS AND BASES.

## I. RAPID ACCURATE DETERMINATION OF AMMONIA OR AMMONIA AND VOLATILE AMINES IN FLUIDS OF BIOLOGICAL INTEREST, AND THE DETERMINATION OF THE DIFFERENT CLASSES OF ACID RADICLES REPRESENTED IN THE TOTAL ALCOHOL TITRATION VALUE.

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In the original account of the alcohol titration method for determining amino-acids [Foreman, 1920] possible developments in regard to its wider usefulness were discussed. It was shown that on applying this method, as if estimating amino-acids alone, to a "synthetic" solution containing the amino-acids of proteins, free acids, salts of ammonia and salts of primary, secondary and tertiary amines, an accurate collective measure of all the acid radicles (termed the "total alcohol value") including all the carboxyl groups save that of arginine is obtained, the latter being quantitatively neutral in the titration. At the same time it was explained why a correct estimate of the total acid radicles value of such a solution cannot be obtained by the Sørensen-formaldehyde titration method, which is the only other existing method available. Briefly, the Sørensen-value would be deficient mainly because the methylene derivatives of secondary amines show basicity to phenolphthalein to an extent varying widely in different individual cases and tertiary amines remain intact. Consequently salts or "internal salts" of secondary amines respond only partially and salts of tertiary amines not at all. It will be shown however that comparisons of alcohol- and Sørensen-values made in the light of such findings may lead to useful deductions.

It was recognised that if the results of applying the alcohol method to fluids of biological interest, which are generally very complex in composition, should be found to represent exclusively the classes of acid radicles mentioned,

a very valuable new analytical figure would be available. Thus for the first time it would be possible to check the sum of individually determined values for the different classes against a reliable total value.

On treating biological fluids with alcohol, sufficient to yield the concentration demanded in the amino-acid method (p. 211), precipitates usually form and clear liquors are obtained. These are referred to throughout as "alcohol precipitates" and "alcohol extracts" respectively. Provided certain adjustments of the original fluids are made in extreme cases [see Foreman and Graham-Smith, 1928, 1, Appendix] the "alcohol extracts" contain the whole of the simple constituents to be estimated. After much investigation it was apparent that the "total alcohol values" of "alcohol extracts" may be regarded as composed entirely of the component values of the classes of acid radicles stated. It will be shown that these total values may be differentiated into sub-values for the classes by rapid methods which avoid undesirable changes in any of the constituents, and that by balance sheet processes the results may be checked. Further, that estimates for certain classes, not hitherto attainable by any direct or indirect means, are obtained as "difference values" which, owing to the reliability of the "total alcohol value" and other direct measurements, are satisfactorily quantitative. By the aid of a special device the "alcohol precipitates" may also be examined in the same manner when considered advisable.

For convenience, a brief account of the investigations into the character of the "total alcohol value" precedes the descriptions of the methods.

*The representative character of the "total alcohol value."*

A general enquiry into the behaviour of "free" bases or their salts when titrated in alcoholic solutions, embracing as many types as possible, was made and various commonly occurring constituents of biological fluids were investigated. The results will be fully discussed in another paper. The findings, so far as they are concerned with the present issues, may be summarised as follows.

*Bases.* Among the bases so far examined, only those in the "strong" category, namely guanidine, quaternary ammonium bases (tetramethyl and tetra-ethyl ammonium hydroxides and choline) and the hydroxides of the alkali metals and alkaline earths, neutralised acids to phenolphthalein in alcohol, all requiring precisely one equivalent. All the other bases tested with this indicator, including ammonia and the three types of amines, were neutral in concentrations of alcohol exceeding about 85 % by volume. Creatinine and the $—CONH_2$ groups of amides were neutral in alcohol as in water. Apparently "strength" determines the titratability in alcohol. It seems very remarkable, however, that none of the many bases examined titrated partially to phenolphthalein in 85–87 % alcohol. In all cases either quantitative titratability or quantitative neutrality was shown.

*Carboxyl derivatives of bases.* When derivatives of those two types of organic bases which neutralised an equivalent of acid in alcohol, namely, guanidines and quaternary ammonium bases, contain a carboxyl group, *e.g.* creatine and betaine respectively, it seems to follow that the carboxyl, as in water, neutralises no alkali on titrating to phenolphthalein in alcohol. Assuming this neutrality to be due to some kind of "internal combination" between the basic nucleus and the carboxyl, and not that the carboxyl is inherently too "weak" an acid to affect this indicator, it follows that the "internal compounds" behave in alcohol quite differently from the "internal salts" of parallel derivatives of much "weaker" bases, namely the amino-acids. The two types of base mentioned and those of their derivatives which were examined, titrated to phenolphthalein in aqueous formaldehyde exactly as they did in 87 % alcohol.

*Acids.* Acids such as carbonic, uric, hippuric, nucleic, lactic, fatty acids, etc., titrate quantitatively as monobasic acids and orthophosphoric as a dibasic acid to phenolphthalein in alcohol. The colour changes are much sharper and the results are more satisfactory, generally, in alcohol than in water. For example, aqueous solutions of pure sodium oleate were appreciably alkaline to phenolphthalein. On adding neutralised alcohol up to 87 %, however, the resulting solutions were precisely neutral, probably due to the alcohol either causing a slight increase in $K_a$ or preventing hydrolysis.

*"Weaker" acids.* The "internal salt" carboxyls of some of the amino-acids give low results in "Stage 2" of the alcohol method but the deficits are made good on completing "Stage 3" giving the "total alcohol value." The carboxyls of peptides are also quantitatively embraced. It should be noted that Stages 2 and 3 both involve titrating to $p_H$ 8·5–8·7.

Judging mainly on the behaviour of the meat extract type of fluid, acids titrating partially to $p_H$ 8·5–8·7, in 87 % alcohol, excepting those amino-acids giving low results which, however, titrate correctly to this $p_H$ on completing "Stage 3," are not present in sufficient quantity to influence "total alcohol values" appreciably. In the first place, the "Stage 3" end-point colour change, adopting the improvement mentioned later (p. 211), is usually very sharp, and, secondly, the small increase shown on carrying a "Stage 2" titration past its end-point to about $p_H$ 9·8 generally corresponds satisfactorily with the sum of the deficits shown by the feebler amino-acids and the early partial value which the acid $HX_2PO_4$ exhibits on titrating to this $p_H$. In the case of ox heart extracts which had been concentrated on the water-bath, and from which the phosphates had been removed, the quantity of alkali required to produce this rise of 1·2–1·3 in the $p_H$ of the alcoholic liquids was extremely slight.

It is important to note that salts which guanidines, quaternary ammonium bases and hydroxides of alkalis and alkaline earths form with organic acids, bicarbonates and secondary phosphates of such "strong" bases, as well as

substances such as creatinine, creatine, betaine and amides, are devoid of any influence in the "total alcohol value." The definite behaviour in the alcohol titration of biological constituents in general suggested that the information gained from a successful differentiation of the "total alcohol value" into its class values, especially if quantitative eliminations were achieved in the processes, might render intelligible the results of titrating subsequently to indicators more acidic, and enable estimates for substances or classes lying outside the value to be made [see "creatinine," Foreman and Graham-Smith, 1928, 1, pp. 81–84].

It is fortunate that we possess in phenolphthalein an indicator so suitable. Harris [1923] however modifies the amino-acid method by titrating to thymolphthalein in alcohol, presumably to $p_H$ 9·9, instead of titrating in the alcoholic formaldehyde to $p_H$ 8·5–8·7 as directed. At the same time he shows that, in addition to the COOH, 28 % of the OH of tyrosine also titrated to thymolphthalein. Other hydroxyamino-acids require testing. Possibly their OH groups may be found to behave similarly. Biological fluids usually contain considerable quantities of phosphates, and on titrating to so high a $p_H$ the acid $HX_2PO_4$ would come into play to some extent. Since none of these very feeble acids influence the "total alcohol value" it will be evident that, especially in the wider applications, as well as in the case of amino-acid mixtures containing hydroxyamino-acids, Harris's modification is not so satisfactory as the original method. Further, inferences based on the relative sizes of "Stage 3" increments and corresponding "Stage 2" values [Foreman, 1920, p. 458], and of alcohol- and Sørensen-values, strictly comparable because obtained by titrating to the same $p_H$, have proved of great assistance. Thymolphthalein proved practically useless in solutions containing formaldehyde.

*Preparation of "alcohol extracts."*

A measured quantity of the fluid to be examined is placed in a measuring flask of ten times its capacity and rectified spirit (97 % alcohol by vol.) of known acidity is added gradually whilst gently rotating the flask so that the precipitate forms slowly. The flask, at once tightly closed with a rubber stopper, is inverted two or three times for thorough mixing and allowed to stand until the supernatant liquid, containing 87–88 % alcohol by volume, is clear. "Alcohol extracts" and "alcohol precipitates" are thus obtained.

*"Total alcohol value" estimations.*

An aliquot portion of the "alcohol extract" is removed without disturbing the precipitate and the stopper tightly replaced without delay. This is immediately titrated as if containing amino-acids alone, as directed [Foreman, 1920, p. 458], utilising an improvement introduced since for obtaining the "Stage 3" value very sharply, as follows. 0·7 cc. of a 25 % aqueous solution of pure NaCl per 50 cc. of alcohol present is added *after completing* the "Stage 2"

titration. The salt causes the sharp definition without appreciably affecting the quantitativeness of the result. The "blank" corrections are then made. It has been found advisable to keep the phenolphthalein solution (0·5 % in 50 % alcohol) with as little exposure to the air as possible.

Accurate carbonate-free aqueous $N/10$ sodium hydroxide (standardised to phenolphthalein) may be used instead of $N/10$ alcoholic potassium hydroxide if sufficient extra alcohol be added to insure that a minimum of 87 % alcohol is present at the close of the "Stage 2" titration.

*Differentiating the "total alcohol value" into class values.*

Strong alcohol was found to exhibit, compared with water, a very feeble capacity to hold volatile bases in solution on raising[1] the temperature. Provided the concentration of alcohol was sufficiently high the passage of a current of steam very rapidly effected their complete removal. Alcohol also passes over and the temperature of the liquid of course rises in relation to the decreasing alcohol percentage. By the time the removal of the bases is complete the liquid is not quite alcohol-free. Therefore even at the close the temperature does not reach 100°.

As already stated such "weak" bases are neutral to phenolphthalein in concentrations of alcohol exceeding 85–87 %. Apparently alcohol at such strength completely dehydrates their hydroxides with the result that there is practically no ionisation. Probably for this reason strong alcohol, unlike water, so readily parts with even the last traces of these bases.

The differentiation of the "total alcohol value" of an "alcohol extract" into class values was rendered possible owing to the fact that the volatile bases are freed from their salts and may be completely removed by means of the rapid steaming process without the necessity of adding alkali in excess of the previously determined "total alcohol value." Since only alcohol and volatile bases are expelled, the liquid retains its quantitative character in respect of the alkali salts of the alcohol-titratable acid radicles, and this enables the further determinations involved in the differentiation to be made.

Only in the volatile bases method of the scheme (including the $CO_2$ elimination, p. 217) is the temperature of the solution raised above that of the room, and in this case the mean temperature over the very short period is considerably below 100°. At the commencement of the distillation the liquid contains no "free" strong alkali and during the distillation the environment of the dissolved substances is alcoholic and not aqueous. Consequently the differentiation is effected under conditions favourable for avoiding the occurrence of undesirable changes in the organic constituents of the "alcohol extracts." Even urea, so easily decomposed in hot aqueous solutions especially if "strong alkali" is present, remains intact in the volatile bases process (p. 220).

[1] Volatile bases in very low concentration apparently show no tendency to escape from strong alcohol at room temperature. It is intended to test the effect of aerating at varying temperatures.

## Description of the methods.

*The rapid method for estimating ammonia or ammonia with volatile amines.*

An aliquot portion of "alcohol extract" possessing a "total alcohol value" of about 5 cc., withdrawn as described (p. 211) is placed in the 500 cc. Jena flask (*A*) and accurate carbonate-free alkali exactly equal to the "total alcohol value" (previously determined) plus the precise amount required to neutralise all alcohol present, and 4 drops of the phenolphthalein solution per 50 cc. alcohol, are added. If aqueous instead of alcoholic alkali is used (p. 212) the extra alcohol required to produce 87–88 % by volume should also be added. For the removal of the bases to be complete the volume of the liquid before commencing the distillation should be at least 75 cc.[1] and should contain at least 87–88 % alcohol. The higher the alcohol concentration the better. Therefore if the volume is less than 75 cc. the deficit is made good with 97 % alcohol.

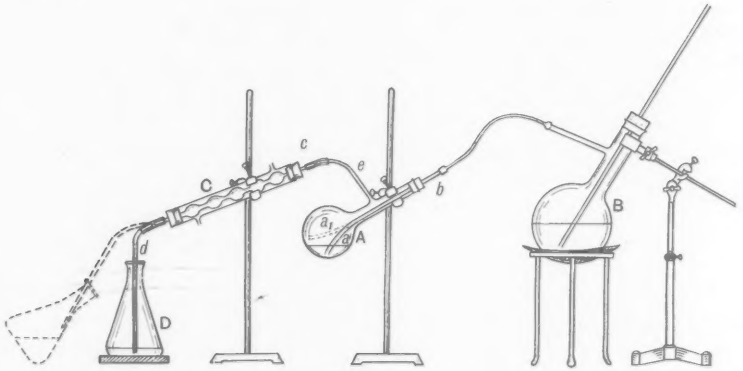


Fig. 1.

"Alcohol extracts" of biological fluids practically always contain small quantities of amino-acids. When amino-acids are present, it is not necessary to add more than the "Stage 2" equivalent of alkali, since a little free alkali is regenerated from their carboxylates during the removal of the bases (p. 215). Consequently in such cases, the alkali addition and the "Stage 2" determination are very conveniently done at one and the same time by a direct titration in flask (*A*)[2]. Opportunities for determining "Stage 3" occur later. Owing to the presence of formaldehyde used in "Stage 3," the solution resulting from a "total alcohol value" estimation should not be employed for the volatile

[1] In trials with accurate *N*/10 $NH_4Cl$ very satisfactory results were given when the volume distilled was 55 cc., but since alcoholic instead of aqueous *N*/10 alkali was used, the percentage of alcohol was higher than 88.

[2] Even when this titration was carried out in a separate vessel and the liquid transferred to flask (*A*) no difference in the volatile bases result could be detected. As little exposure to atmospheric $CO_2$ as possible, however, is desirable.

bases process. A slight turbidity sometimes appears at the close of a "Stage 2" titration. The result, however, is not affected. The turbidity disappears immediately the distillation commences.

In the 2 litre flask (*B*) steam is generated from *distilled water* previously boiled gently for $\frac{3}{4}$ hour to remove $CO_2$ and traces of ammonia. If kept hot, $1\frac{1}{2}$ litres thus prepared serves for several estimations.

The tubing throughout is 5–6 mm. internal diameter. It is important that the lower end of tube (*a*) should not be drawn out but simply cut off at a right angle, and that the junction between (*c*) and (*e*) should be made as sketched. A condenser whose inlet is adapted to receive a rubber stopper into which tube (*e*) must be rigidly fixed is unsuitable. Much convenience will be sacrificed if attempts are made to use the condenser in an upright position. When disconnecting flask (*A*) the original tight fixture of the rubber connector on tube (*c*) should not be disturbed.

The distillation should be commenced as quickly as possible after adding the alkali. The following steps are therefore taken before preparing the alcoholic liquid. (1) Tube (*a*) with the rubber stopper (*b*) fixed firmly in its right position, withdrawn from flask (*A*), is attached to the steam generator and the water brought to a vigorous boil so that a rapid jet of steam issues. This jet may be conveniently used, in the meantime, for giving an extra wash to the condenser. (2) 5 cc. *N*/10 HCl or an equivalent quantity of weaker standard HCl, sufficient to provide excess, is placed in the 750 cc. Erlenmeyer flask (*D*) and $CO_2$-free distilled water is added to give a layer deep enough to cover the end of tube (*d*) satisfactorily. Flask (*A*) and the receiver are then connected up and tube (*a*), with the steam jet issuing, is quickly inserted and fixed.

Alcohol containing the volatile bases is expelled so rapidly that it passes down tube (*d*) almost as if poured. The removal of bases is complete by the time a foam rises uniformly above the surface of the liquid. The time occupied in obtaining the foam depends upon the volume of liquid to be distilled and its percentage of alcohol. 75 cc. containing 87–88 % alcohol requires about 5 minutes and 100 cc. $5\frac{1}{2}$–6 minutes. The end of tube (*a*) is then raised above the surface by giving the stopper of flask (*A*) a half-turn so that the bend occupies the position ($a_1$), and the interior is swept by further steam for one minute. The operation is then concluded by raising the cork of the steamer.

If the "alcohol extract" contains plenty of fatty acid radicles the foam may rise so suddenly that, unless closely watched it may gain access to the condenser. After allowing the foam to approach the neck of the flask, however, its further rise may be instantly checked and its rapid disappearance effected by raising the cork of the steamer. The one minute sweep is then applied. In other cases the foam may rise so little as possibly to escape detection[1]. Use is

[1] When estimating volatile bases only the foaming may be accentuated by adding a drop or two of strong castile soap solution or some other foam producer before the steaming. If, however, a "regenerated alkali" measurement (p. 215) is desired, the foam producer used should be quite neutral in that titration.

then made of the observation that at the foaming stage water will collect as separate drops in tube (*e*) if this is of the right size (p. 214). However, in order to make certain that the operation is not stopped prematurely the steaming may usually be continued for one minute longer[1] than the periods mentioned above, followed by the one minute sweep.

The residual liquid is examined before titrating the distillate.

*The distillate.* Receiver (*D*) is tilted and withdrawn to the left so that the end of tube (*d*) is well clear of the liquid. If withdrawn sufficiently it will be held securely in an inclined position as sketched. The condenser is then thoroughly washed down into the distillate with $CO_2$-free water and the excess of HCl titrated with *N*/10, *N*/20 or *N*/50 carbonate-free alkali to alizarin red[2]. It is important not to add too much alizarin red. 0·05 to 0·1 cc. of a filtered 1 % aqueous solution, or 2 drops from a fine dropper, producing a light greenish-yellow colour in the solution, is ample. In such dilutions it is extremely sensitive to free alkali, as little as one drop of *N*/50 solution causing an abrupt change to brown or brownish-red. The colour change is not quite so sharp on titrating alkaline solutions with HCl, and in such cases it is better to add excess of HCl and titrate back. It is equally sensitive in either alcohol or water.

*The residual liquid.*

(1) *Estimation of "regenerated alkali."* As the liquid becomes aqueous, owing to the expulsion of alcohol by the steam, the non-volatile amines regain their basicity to phenolphthalein which they had completely lost in the 87–88 % alcohol, and the sodium salts of the "internal salt" carboxyls of the amino-acids hydrolyse to yield their equivalent of free alkali. The alkali salts of all the other alcohol-titratable acid radicles are neutral in the water as they were in the alcohol. The total alkalinity of the residual liquid, termed the "regenerated alkali" therefore represents the sum of the values for these two classes.

Flask (*A*) with its stopper in position is disconnected and immediately cooled under the tap, imparting only a slow rotatory movement to the liquid so as to expose it as little as possible to atmospheric $CO_2$. Without delay, the stopper containing tube (*a*) is raised, the tube washed down with a little water, and the red liquid titrated in the flask over a white plate with *N*/10 HCl until only faintly pink. Four more drops of the phenolphthalein solution are then added and the addition of acid continued until one drop removes all traces of pink colour. This last drop is not included in the reading. Or—probably the better way—after adding the 4 drops of indicator solution, a slight excess of *N*/10 HCl is added and the liquid titrated back with *N*/10 alkali until one drop produces *the faintest possible* pink colour. One drop more of the indicator

[1] In the case of meat extracts trials showed that even when continued 1½ minutes longer exactly the same result was obtained. Other experiments showed that even when such an easily decomposable substance as urea is present the steaming may be continued ½ to ¾ minute past the foaming stage.

[2] Miscalled "alizarin" in the preliminary description [Foreman, 1920, p. 470].

solution generally facilitates this final adjustment. The residual liquid is thus titrated to $p_H$ 8·3–8·4. This is not difficult since, apart from the indicator colour, the liquids are clear and generally colourless.

In a series of careful "blank" estimations, steam-distilling 55–75 cc. of neutralised 88 % alcohol to which known quantities of $N/10$ sodium hydroxide had been added, the residual liquids, titrated as described, consistently required 0·1 cc. less than the equivalent of $N/10$ HCl, probably due to traces of $CO_2$ derived from the steam. The true "regenerated alkali" value is, therefore, given on increasing the actual reading by 0·1 cc. The distillates neutralised much less than one drop of $N/20$ HCl, showing the "blank" volatile bases value to be almost negligible.

*Discussion.* The standard light pink end-point adopted in all phenolphthalein titrations [Foreman 1920, p. 460] corresponds to about $p_H$ 8·7. In aqueous solutions titrated with alkali to this $p_H$ the "internal salt" carboxyls of some of the amino-acids are dissociated as the sodium salts to the extent of about 4 %, while, on the other hand, titrated to this $p_H$ with HCl, amines are generally not quite completely dissociated as the hydrochlorides. Further, the residual liquids, especially if distilled only as far as the foaming stage, contain a small percentage of alcohol, possibly reducing the basicity of the amines very slightly. Consequently the "regenerated alkali" titrated is carried to $p_H$ 8·3, the colourless stage of phenolphthalein, yielding, as shown later, results which fit satisfactorily in balance sheets.

(2) *Estimation of the "internal salt" carboxyls of amino-acids.* The neutral solution resulting from the "regenerated alkali" estimation is titrated by the Sørensen-formaldehyde method to the light pink end-point (see above). 50 cc. of formaldehyde solution made by diluting *good* formalin with 1½–2 vols. of water provides sufficient excess.

(3) *Estimation of non-volatile amines.* The difference between the "regenerated alkali" value and the Sørensen value of the neutralised residual liquid gives an approximate value for the non-volatile amines, an estimate not hitherto attainable.

*Discussion.* It is insufficiently recognised that Sørensen values obtained after eliminating volatile bases may include the values of acid radicles in salts of any non-volatile primary amines and the partial values of acid radicles in salts of any non-volatile secondary amines which may be present (see p. 208). Unfortunately no method at present exists for obtaining a closer approach to the true amino-acid value when amines are present. Perhaps the Van Slyke nitrous acid method might be applied with some advantage, but only the primary amine radicles in amino-acids respond, and amines, *e.g.* the diamine putrescine, do not all fail to yield nitrogen promptly in that process. Amides should also be considered as another possible source of Van Slyke-nitrogen. Back titration with HCl to methyl red after titrating in alcohol, as directed by Harris [1923, p. 508] for amino-acids, would embrace amines as well and therefore affords no help.

In the case of one fluid at any rate, namely sterilised ox heart extracts, it seemed evident that salts of the non-volatile amines possessed little or no Sørensen value and that the Sørensen value of the neutralised residual liquid represented the amino-acids fairly satisfactorily. [See Foreman and Graham-Smith, 1928, 1, pp. 66–68.]

(4) *Estimation of the "net acidity" of "alcohol extracts."* Having applied the blank corrections, the sum of the values for the volatile bases (also representing the acid radicle value of their salts) and the "regenerated alkali" (equivalent to the sum of the acid radicle values of the salts of non-volatile amines and the "internal salt" carboxyls of the amino-acids), subtracted from the "total alcohol value," gives a value termed the "net acidity" which affords an indirect measure of what would be the free acid value of the constituents of the "alcohol extracts" if they were in aqueous instead of alcoholic solution.

The differentiation into class values is thus completed.

*Elimination and estimation of the* —$HCO_3$ *radicle in "alcohol extracts."*

A full account of this room temperature process is given in the following paper [Foreman, 1928]. By its aid, in addition to obtaining the —$HCO_3$ estimate, the accurate "Stage 2" value of acid radicles other than the carbonic radicle may be determined, and this net value may be differentiated into class values on applying the foregoing methods to the resulting solution, with the advantage that the results are more precise.

*Checking the class value results by balance sheets.* The acidity value of the "alcohol precipitate" can be very satisfactorily determined by titrating its filtered aqueous extract to phenolphthalein, the end-point as a rule being quite sharp. Experience has shown that whenever an original fluid contains too little pigment or turbidity to affect the precision of its direct titration to phenolphthalein, the resulting acidity value agrees remarkably closely with the sum of the "net acidity" value of the "alcohol extract," and the acidity value of the "alcohol precipitate." Since the accuracy of the "net acidity" value, which is a balance value, depends upon the precision of the "total alcohol value" and other actual measurements, such agreement could not occur consistently if these measurements were unreliable. The results are, therefore, checked by observing the closeness of this agreement.

The preparation of aqueous extracts of "alcohol precipitates," and the means for obtaining as accurately as possible the direct phenolphthalein value of the original fluid when pigment is a little too much in evidence, are described elsewhere [Foreman and Graham-Smith, 1928, 1, p. 12 and p. 6].

Although pigment such as that seen in highly concentrated meat extracts which have been heated seems to be completely removed in the "alcohol precipitate,"[1] yet the clear water extract of this precipitate shows practically no

[1] On the other hand "alcohol extracts" made from unheated aqueous extracts of green vegetable matter do generally contain a good deal of pigment, but it was found that these alcoholic

pigmentation. The greater sharpness of the phenolphthalein colour change in this extract than in the original fluid is probably due partly to the fact that the "weak" nitrogenous bases of the fluid are not present. Such bases, especially if not present in small quantities, do not titrate very sharply to this indicator in water. On the other hand they do not prevent precise titrations in 87 % alcohol because they display no basicity to phenolphthalein in this solvent. Consequently it is believed that the sum mentioned affords a more accurate estimate of the acidity of the whole fluid than a direct titration, especially if there is pigment interference in determining the end-point in the latter. In practice a discrepancy between the sum and the direct value can nearly always be reasonably attributed to pigment effects in the direct titration, and in series the sums generally vary more consistently with $p_H$ fluctuations in the fluids. Limited evidence at present available shows that reliable acidity values for fluids too turbid or highly pigmented for a direct acidity titration to be of any value may be estimated in this indirect manner.

*Estimation of volatile acids.* These are estimated in portions of the "alcohol extracts," after applying the $CO_2$ elimination process if carbonic acid is present [see Foreman, 1928, p. 225], by steam-distilling under standard conditions as described [Foreman and Graham-Smith, 1928, 1, p. 32].

*Estimation of non-volatile acid radicles.* A "difference value" for the non-volatile acid radicles (not including those which may be present in salts of "strong" bases, see p. 209) is estimated by subtracting from the "total alcohol value" of the solution resulting from a $CO_2$ elimination (p. 217), the sum of the values for the "internal salt" carboxyls of the amino-acids and the volatile acids. A reliable estimate for this class of acid radicles has not hitherto been possible.

*Alcohol titration values of "alcohol precipitates" and their differentiation into class values.* Provided the "alcohol extracts" in a series of examinations are prepared in a standard manner [see Foreman and Graham-Smith, 1928, 1, Appendix] the class value fluctuations will reflect changes satisfactorily and

liquids may be practically decolorised by shaking in the cold with very small quantities of Merck's medicinal charcoal and that, provided the liquids are acid, there is no adsorption of the important index constituents. "Alcohol extracts" from plant and animal tissues are always acid since, apart from any free acids in the original aqueous fluids, the acid radicles in salts of ammonia and amines as well as the "internal salt" carboxyls of amino-acids titrate to phenolphthalein as acids. The alcohol treatment has not yet been applied to unheated aqueous liquids containing free haematin, but it seems very likely that if the "alcohol extracts" are found to be too red, the charcoal treatment will prove equally effective. Unheated water extracts of animal and fish tissues gave "alcohol extracts" satisfactorily free from pigment.

The following method also proved very satisfactory in the case of the juice from green mangel leaves. 50 cc., after filtering through cotton wool, was made up to 100 cc. with 97 % alcohol and some sludge filtered off. The clear liquor was shaken in the cold with the minimum quantity of Merck's charcoal required to remove the green colour and filtered. 20 cc. of the filtrate, possessing a very faint brownish tinge, was made up to 100 cc. with 97 % alcohol, giving an ordinary "alcohol extract" perfectly colourless, and a white "alcohol precipitate." "Alcohol precipitates" generally begin to appear when the alcohol concentration reaches about 40 %. Perhaps alcohol added to the juice up to 40 %, instead of 50 %, would have sufficed; also less chlorophyll would have dissolved.

in many cases further examination of the "alcohol precipitates" will be unnecessary but, if desired, and to cover other cases, the class values of the aqueous extracts of these precipitates may be determined as follows.

*The alcohol-glycerol method.* It was found that many substances insoluble in 87 % alcohol are not precipitated if sufficient glycerol is mixed with their aqueous solutions before adding the alcohol, and that while thus held in solution their alcohol titration values may be determined. The constituents in aqueous extracts of "alcohol precipitates," chiefly phosphates, complex protein derivatives, peptides, etc., respond in this manner and the methods for estimating volatile bases, "regenerated alkali" and "net acidity" are applicable to the solutions resulting from the titrations exactly as described for "alcohol extracts."

A sufficiently concentrated filtered aqueous extract is made (p. 217) and a 5 or 7 cc. aliquot part possessing a "Stage 2" value of 1·5–3 cc. $N/10$ solution is placed in flask *A* (Fig. 1), an equal volume of pure glycerol, measured in a cylinder without allowing it to fall on the sides, is drained into the flask without contaminating the neck. The remaining glycerol is washed in with 97 % alcohol, sufficient to yield 86 % after the titration which follows, added gradually with further mixing. The solution resulting from the "Stage 2" titration is then examined exactly as described for "alcohol extracts." The "blank" value is somewhat larger than the acidity of the spirit used. If any turbidity appears the result is unaffected since the glycerol prevents its flocculation and it practically all dissolves as the titration proceeds. The results are checked by observing the closeness with which the "net acidity" agrees with the direct acidity value of the aqueous extract.

Class values for fluids as a whole may be determined after glycerol treatment, but, except in the case of the volatile bases, pigments are likely to interfere too much in the decisions. Further, since class values for each of the two alcohol fractions may be obtained in under 10 minutes, with numerous advantages, this course is not advised.

No biological fluid has yet been encountered containing weak acids resembling boric acid in exhibiting an increase in strength on adding glycerol. Even amino-acids are not appreciably affected. For example, the "free" carboxyl and the HCl of glutamic acid hydrochloride in an aqueous solution containing about 40 % of glycerol titrated to phenolphthalein exactly as if in water alone, save that perhaps the end-point was a little more satisfactory. Consequently the strength of the "internal salt" carboxyl was practically unaffected.

*Application to culture media.* A demonstration of the usefulness of these methods and how they are adapted to meet special circumstances, is provided in the full account of the investigation of the changes produced by *Staphylococcus aureus* in meat extracts [Foreman and Graham-Smith, 1928, 1, 2].

*Application to urine.* So far only one specimen of fresh urine has been examined partially. Much pigment was present and the constituents were in

high concentration. For estimating the acidity a portion was diluted with five volumes of water. The "alcohol extract" was practically colourless. The "alcohol precipitate" was completely soluble in water. After correcting for "blanks," the following results—cc. $N/10$ alkali or acid per 5 cc. urine—were obtained:

| "Alcohol extract" | | | | | | "Alcohol precipitate." | | |
|---|---|---|---|---|---|---|---|---|
| "total alcohol value" | volatile bases | non-volatile amines | amino-acids | "net acidity" | | Aq. extr. titrated to pp. | Sum of columns 5 and 6 | Untreated urine titrated to pp. |
| (1) | (2) | (3) | (4) | (5) | | (6) | (7) | (8) |
| 5·25 = | 2·68 + | 0·49 + | 0·38 + | 1·70 | | 0·24 | 1·94 | 2·01 |

The distillation of volatile bases was continued one minute past the foaming stage (see below). Apparently, since urines vary widely in their content of dry matter, the phosphates would be in differing concentration and detailed examination of the "alcohol precipitates" would be necessary (see p. 218). Further investigation is needed.

*The behaviour of urea in the volatile bases process.* Simulating an average specimen of urine, 2·5 % aqueous solutions of (*a*) a specimen purchased as "pure urea" and (*b*) the product of crystallising (*a*) from water, were examined. Both solutions were practically neutral to phenolphthalein and 5 cc. portions possessed corrected "total alcohol values" of 0·13 and 0·18 cc. $N/10$ solution respectively. 5 cc. of solution (*b*) + 50 cc. 97 % alcohol + 1·0 cc. $N/10$ sodium hydroxide (a little more than the "regenerated alkali" value shown above in columns 3 + 4) gave 0·08 cc. $N/10$ volatile bases.

10 cc. of a 5 % solution of a less reliable specimen + 100 cc. 97 % alcohol + 4 cc. $N/10$ sodium hydroxide, distilled one minute past the foaming stage gave 0·33 cc. $N/10$ volatile bases and 3·6 cc. of free sodium hydroxide in the residual liquid.

It seemed evident, especially since urea completely decomposed yields $NH_3$ and the $—HCO_3$ radicle in the ratio of 2 : 1, that the volatile bases in the distillates were derived from salt impurities, probably ammonium salts, and that, in spite of the presence of the small quantities of free sodium hydroxide[1], the urea practically remained intact.

Subsequently it was found that after specimen (*a*) had been thrice crystallised from water the alcohol value reached zero and, treated as in the case of (*b*) but with 2–3 % more alcohol present, no increase over the "blank" distillate value could be detected on distilling half-a-minute past the foaming stage. In a similar trial distilling one minute past the foaming, however, the distillate showed very faint indications of derived ammonia. These observations confirm those of Levy-Simpson and Carroll [1923, p. 396]. When dealing with fluids containing urea it is therefore inadvisable to pass the foaming stage by more than 30 to 45 seconds.

[1] It should be noted that ordinary "regenerated alkali" values are small and only partly due to free sodium hydroxide (p. 215).

In the meantime the methods here described have been utilised by others as follows.

The volatile bases method by Cole [1926], Halnan [1926], Levy-Simpson and Carroll [1923] and McCance [1924]. The methods from the class values standpoint by Woodman [1925] and, under my supervision, by Miss Marrack [1922].

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# XXXI. FURTHER DEVELOPMENTS OF RAPID VOLUMETRIC METHODS FOR THE DETERMINATION OF AMINO-ACIDS, ORGANIC ACIDS AND BASES.

## II. RAPID QUANTITATIVE REMOVAL AND DETERMINATION OF THE CARBONIC ACID RADICLE, ESPECIALLY IN BACTERIAL CULTURES, WITHOUT EXPOSING OTHER CONSTITUENTS TO RISK OF LOSS OR CHANGE.

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(*Received August 23rd, 1927.*)

In a new method described in the preceding paper [Foreman, 1928] "freed" volatile bases in the "alcohol extracts" of biological fluids are very rapidly expelled by means of a current of steam. It seemed possible that "freed" volatile acids might respond similarly, but on investigation they were found to behave in the directly opposite manner, showing less tendency to escape the higher the alcohol concentration. During the collection of the earliest small fractions of their steam distillates, solutions as highly alcoholic as "alcohol extracts" (87–88 % by vol.) were very highly retentive of "free" volatile acids. As the alcohol percentage fell and consequently as the temperature approached nearer to 100°, the volatile acids, in spite of the progressive reduction in their concentration, passed over at a gradually increasing rate which reached a maximum by the time the liquids were practically alcohol-free.

The phenolphthalein titration values of the first one-minute distillates from 105 cc. quantities of 92 % alcohol containing (*a*) acetic acid to the value 9·0 cc. *N*/10, (*b*) butyric acid to the value 9·43 cc. and from 55 cc. quantities of 88 % alcohol containing (*c*) acetic acid to the value 9·0 cc., (*d*) butyric acid to the value 9·43 cc., were 0·16, 0·17, 0·32, 0·31 cc. *N*/10 respectively. The one-minute periods were timed from when the distillates commenced to run into the neck of the condenser. Series of successive one-minute distillates obtained in the case of (*b*), (*c*) and (*d*) required *N*/10 alkali as follows:

(*b*) 0·17, 0·17, 0·2, 0·3, 0·42, 0·7, 0·7, 0·72, 0·71, 0·6, 0·5 cc.

(*c*) 0·32, 0·6, 0·75, 0·72, 0·72, 0·72, 0·6.

(*d*) 0·31, 0·47, 0·82, 1·2, 1·14, 0·94, 0·69, 0·55.

The condenser was well washed down between each fraction. 105 cc. and 55 cc. of 87 % alcohol lost practically all their alcohol in 5 to 6 and about 4 minutes respectively. In (*b*) the liquid in the flask gained an average of about 8 cc. water per minute as condensed steam.

It will be noted that only very small percentages of the volatile acids present were expelled during the first minute.

50 cc. water containing butyric acid to the value 11·14 cc. *N*/10, warmed nearly to 100° before commencing the steaming, gave the following successive one-minute values:

2·8, 1·35, 0·98, 0·86, 0·7 cc. *N*/10.

A brisk current of steam kept as uniform as possible throughout these experiments was used.

The high retentiveness of 87 % alcohol for volatile acids when steamed, even though the temperature was probably as high as 85°, coupled with another observation that $CO_2$ escapes extremely readily from its partially saturated solution in alcohol on exposure to the air at room temperature, gave rise to the suggestion that at temperatures well below 85°, a current of air passed through a solution of carbonic acid and "free" volatile acids in 87 % alcohol, causing practically no change in the alcohol concentration, might very rapidly remove the $CO_2$ completely and leave the whole of the volatile acids behind. These expectations were fulfilled and a simple rapid room temperature process for completely[1] removing and estimating the carbonic acid radicle in "alcohol extracts" without exposing other constituents (including volatile bases) to risk of loss or change, was evolved.

### Description of the Method.

*Rapid elimination of the carbonic acid radicle from "alcohol extracts."* The "Stage 2" value of the "alcohol extract" to be examined is first accurately determined exactly as if only amino-acids were present [see Foreman, 1928, p. 211]. Another aliquot portion, 25 cc. if plenty, and 50 cc. if little, of the carbonic acid radicle is present, is placed in the 250 cc. Erlenmeyer flask (*A*), and a quantity of accurate *N*/10 HCl equal to the "Stage 2" value added[2] from a burette. The tightly fitting rubber stopper containing tubes (*a*) and (*b*) is then fixed securely in position so that the lower end of tube (*a*) is as near to the bottom of the flask as possible without actually touching. Tube (*a*) is $4\frac{1}{2}$ to 5 mm. internal diameter. Its lower end should be a simple straight cut-off at right angles, and the opening should not be reduced in size. It is attached to the 250 cc. wash-bottles each containing about 50 cc. of 1 in 2

[1] It was found that, in addition to introducing errors in subsequent estimations, precipitation processes, *e.g.* with barium hydroxide, do not eliminate the carbonic acid radicle satisfactorily.

[2] This addition of HCl was originally introduced for liberating carbonic acid from its salts and to fix volatile bases during the $CO_2$ removal. Probably much less would suffice. Later findings indicate that in the great majority of cases the addition of HCl may be dispensed with altogether (see p. 226).

potassium hydroxide. A very brisk[1] current of air, but not so fast that the splashes approach too near tube (*b*), freed from $CO_2$ by the potassium hydroxide, is drawn by means of the pump through the alcoholic liquid for 3 minutes if 25 cc., or 5 minutes if 50 cc., of the "alcohol extract" is used. Tube (*a*) is then closed by clip (*f*) and air admitted *immediately* through tap (*d*). The rubber attachments on tubes (*a*) and (*b*) are then removed, the stopper containing these tubes is raised, and tube (*a*) washed down with 5 cc. rectified spirit from a pipette. Finally the inlet tube of $B_2$ is sealed by clip (*e*) in order to preserve the potassium hydroxide for further estimations.

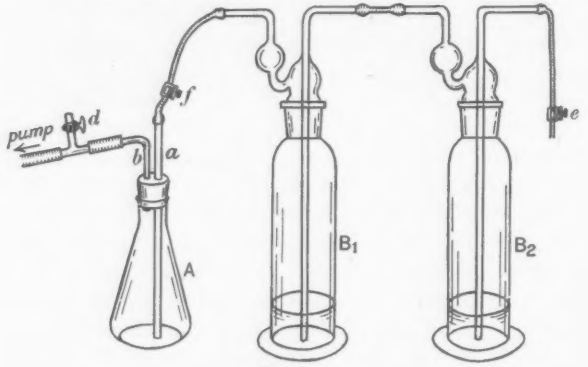


Fig. 1.

The periods mentioned afford ample margins of time for securing the complete removal of the $CO_2$ at room temperature. If flask (*A*) is placed in a water-bath at 40 to 45° while the suction is applied, 3 minutes is more than sufficient even for 100 cc. (see p. 228), but although no loss of volatile acids occurs at this temperature yet, for the sake of saving a very little time, it seems inadvisable to sacrifice the assurance associated with a room temperature process that the other constituents have not undergone change. At the close of an operation if warming has been practised the lower end of tube (*b*) and the base of the stopper may show traces of condensed alcohol. Apparently the lower end of tube (*a*) being so near the bottom causes the splashes to be directed on to the walls of the vessel for about half the way up, the aeration being the more effective the less the depth of the liquid. Perhaps therefore a somewhat larger Erlenmeyer would be more suitable for 50–100 cc. of "alcohol extract."

*Estimation of the remaining alcohol-titratable acid radicles.* After the addition of a quantity of rectified spirit equal to ten times the volume of the *N*/10 HCl originally added, the "Stage 2" value of the alcoholic liquid resulting from the aeration is accurately determined by titrating to phenolphthalein with accurate *N*/10 carbonate-free alcoholic potassium hydroxide (standardised with phenolphthalein as indicator). The "Stage 2" value for

[1] In many successful estimations at room temperature and at 45° aerating about 30 cc. alcoholic liquid the pressure was reduced 50–80 mm. by the suction.

the whole of the alcohol-titratable acid radicles of the "alcohol extract" other than the carbonic acid radicle is given on subtracting the value of the $N/10$ HCl originally added from the reading of alcoholic potassium hydroxide.

This net "Stage 2" value may be differentiated into class values on applying the methods described [Foreman, 1928] to this solution, or the solution may be used for any estimation, *e.g.* volatile acids, with the advantage that, in the absence of the carbonic acid radicle, the results are more precise. Owing to the fact that a little of the $CO_2$ is caught in a volatile acids distillate, it is important, especially when the volatile acids content is small, to eliminate the carbonic acid radicle prior to their distillation.

*Estimation of the carbonic acid radicle.* The difference between the original "Stage 2" value and the net "Stage 2" value after the aeration gives the $—HCO_3$ value of the "alcohol extract." The "blank" value is usually very minute.

*Loss of* $CO_2$ *in preparing and titrating "alcohol extracts."* An advanced culture of bacteria in a highly concentrated unneutralised meat extract contained the $—HCO_3$ radicle at a concentration of about $N/15$. The culture was very nearly neutral to phenolphthalein, showing the $CO_2$ to be practically all in the form of bicarbonate. Examined by the alcohol methods, including the $CO_2$ process, the balance sheets [Foreman, 1928] showed that 15 % of the $—HCO_3$ radicle originally present in the culture had been lost, leaving an equivalent amount of "free" weak nitrogenous bases in the alcoholic liquid. Supplementary experiments showed that this loss was due to the escape of $CO_2$ during the short exposures involved in preparing and pipetting the "alcohol extract" and while agitating it during the "Stage 2" titration, apparently mainly in the titration.

Since the percentage of the total $CO_2$ lost in these exposures would fall to a largely increased extent the higher the degree of dilution, loss may be inappreciable when the carbonic acid content is very small or even when dilute media contain moderate amounts. However, bearing in mind that the advantages of carbonic acid elimination (see above) and perhaps other advantages [see Foreman, 1928, p. 212] are gained by direct applications of the method to ordinary "alcohol extracts," that part of the loss which occurs during the direct "Stage 2" titration, or the total loss if a figure for the whole of the carbonic acid of the culture is desired, may be avoided as follows.

*Avoiding loss of* $CO_2$ *during a "Stage 2" titration.* The *first* aliquot portion of the "alcohol extract" [Foreman, 1928, p. 211], withdrawn by gentle suction in the pipetting, is delivered with the tip of the pipette under the surface of a mixture of $N/10$ alkali sufficient to provide excess with any extra 97 % alcohol which may be needed to give 87 % alcohol by volume. The solution is then titrated back with $N/10$ HCl. The $CO_2$ is thus all fixed as $NaHCO_3$ at the commencement of the titration. This "Stage 2" value instead of that obtained by direct titration is then used in determining the $—HCO_3$ value as described above.

*Avoiding all loss of* $CO_2$ *in estimating the* $-HCO_3$ *value of the culture.* A full account of this process as suggested for highly concentrated cultures has been already given [Foreman and Graham-Smith, 1928, Appendix]. Briefly, a portion of the culture is delivered with the pipette lip under the surface into sufficient $CO_2$-free sodium hydroxide to render just alkaline to phenolphthalein the solution obtained on subsequently adding two volumes of 97 % alcohol. Much undesirable matter with most of the pigment is precipitated in this 65 % alcohol but the whole of the $NaHCO_3$ and volatile acids are left in solution. An aliquot part of this solution is then delivered under the surface into a mixture of 1 cc. $N/10$ sodium hydroxide and sufficient 97 % alcohol to yield 87 % alcohol when the aliquot part is present, and $N/10$ HCl is run in drop by drop until the "Stage 2" end-point is reached. The measured quantity of $N/10$ HCl is then added, the $CO_2$ removed, and the liquor titrated with $N/10$ alcoholic potassium hydroxide, as described for ordinary "alcohol extracts" (p. 223). The difference between the value of the measured quantity of $N/10$ HCl and the reading of $N/10$ alcoholic potassium hydroxide gives, after subtracting the very small $-HCO_3$ value of the sodium hydroxide used, the $-HCO_3$ value of the culture. The same liquid is then used for estimating volatile acids which will be obtained in $CO_2$-free condition.

*Carbonic acid in the form of bicarbonates of weak nitrogenous bases in the aqueous solution behaves like "free" carbonic acid on aerating the "alcohol extract" at room temperature.* The observation in regard to the loss of $CO_2$ from the "alcohol extract" of the neutral culture mentioned on page 225 suggested that "weak" nitrogenous bases had no power to augment the low retentiveness of strong alcohol for carbonic acid, and that it might be possible to eliminate the whole of the carbonic acid radicle by simple aeration without employing mineral acid. Using the apparatus (Fig. 1), a very gentle air current, passed for 10 minutes through 25 cc. portions of the "alcohol extract," at room temperature, completely removed the $CO_2$ and left the whole of the other constituents intact. No loss[1] of volatile bases occurred, estimations made before and after the aeration giving identical results. Unfortunately the effects of brisk air currents (p. 224) were not investigated.

By means of this modification it was found possible to show that ordinary salts of non-volatile amines, and not substances of the nature of amino-acids in possessing "internal salts," were responsible for the considerable extent by which the "total alcohol value" of the "alcohol extract" of this culture exceeded the comparable Sørensen-formaldehyde value [see Foreman and Graham-Smith, 1928, p. 61].

Whether or no the mineral acid may be omitted in the case of "alcohol extracts" of cultures containing "free" nitrogenous bases or of cultures in media neutralised with sodium hydroxide before inoculation, requires

[1] Since weak nitrogenous bases show no basicity to phenolphthalein in the "Stage 2" titration, loss of volatile bases during the aeration would not affect the $-HCO_3$ estimate. Loss of volatile bases, however, would vitiate certain subsequent estimations (p. 225).

investigation, but it seems possible that provided a sufficiency of alcohol-titratable acid radicles (other than the carbonic acid radicle) is present, the $[H^+]$ of the "alcohol extracts" may be high enough for a complete removal of $CO_2$ at room temperature without loss of volatile bases. The HCl addition, however, would be necessary in the case of "alcohol extracts" made from aqueous solutions of the carbonates or bicarbonates of "strong" bases alone or in the presence of less than an equivalent quantity of alcohol-titratable acid radicles.

Cultures fairly acid to phenolphthalein which have been incubated at 37° usually contain only traces or very small quantities of carbonic acid. In these cases, since $CO_2$ estimations are unimportant, it is now customary to introduce aliquot portions of the "alcohol extracts" to be used for other estimations straight into the apparatus and, without additions of any kind, to aerate gently for a few minutes at room temperature.

## Experiments.

Using the apparatus (Fig. 1), brisk air currents were drawn for differing periods through mixtures of 97 % alcohol, a half-saturated solution of $CO_2$ in 97 % alcohol and known quantities of standard solutions of common volatile acids, at different temperatures. The results are shown in the following table:

Table I.

| Acid | 97 % alcohol used cc. | ½ sat. alcoholic $CO_2$ added: quantity cc. | ½ sat. alcoholic $CO_2$ added: $-HCO_3$ value cc. $N/10$ | Aqueous acid added cc. | % alcohol in mixture | Suction period minutes | Temp. | Pp. value of aqueous acid added cc. $N/10$ NaOH | Result after allowing for spirit acidity cc. $N/10$ alc. KOH |
|---|---|---|---|---|---|---|---|---|---|
| Acetic | 25 | 5 | 2·15 | 0 | — | 2 | room | 0 | 0·05 |
| | 25 | 5 | ,, | 5 | 83 | 2 | ,, | 9·0 | 9·0 |
| | 25 | 5 | 2·85 | 0 | — | 2 | 50° | 0 | 0·02 |
| | 25 | 5 | ,, | 5 | 83 | 2 | 46° | 9·0 | 8·99 |
| Formic | 25 | 5 | 3·45 | 5 | 83 | 2 | room | 9·05 | (*a*) 9·15 (*b*) 9·05 |
| | 25 | 5 | ,, | 5 | 83 | 2 | 45–50° | 9·05 | 9·05 |
| Butyric | 25 | 5 | about 3·0 | 5 | 83 | 3 | room | 9·43 | 9·43 |
| | 25 | 5 | ,, | 5 | 83 | 3 | 40–45° | 9·43 | 9·40 |
| | 50 | 5 | ,, | 5 | 89 | 3 | room | 9·43 | 9·40 |
| | 25 | 0 | — | 5 | 81 | 5 | ,, | 9·43 | 9·43 |
| | 25 | 0 | — | 5 | 81 | 5 | 40–45° | 9·43 | 9·38 |
| | 30 | 0 | — | 0 | — | 5 | ,, | 0 | 0·02 |
| Hydrochloric | 25 | 5 | 3·45 | 6 | 81 | 2 | room | 6·0 | 6·03 |

A comparison of the figures in the last two columns shows that the $CO_2$ is completely removed in a very short time and that even at about 45° the volatile acids, in such concentrations, are completely retained. Perhaps traces of butyric acid were removed in 5 minutes at 40–45° from 30 cc. of 81 % alcohol (see table), but having regard to the very small percentage lost in the first minute of steaming (p. 222) this inference seems hardly justifiable. It may be mentioned that a mixture exactly as in the fourth acetic acid experiment which had been gently moved for 2 minutes in the open vessel placed in water at 90° (no suction) titrated 8·98 cc. (cf. 9·0 cc. for the acetic acid used).

Various tests of a similar nature were applied to an old "alcohol extract" of an advanced putrefactive culture. Since it contained nitrogenous bases it was important to take care that the requisite minimum of 86 % alcohol was present at the close of the titrations, and it proved convenient to use $N/10$ alcoholic potassium hydroxide. Mixtures of 10 cc. portions of the "alcohol extract," 50 or 100 cc. 97 % alcohol, 10 cc. half-saturated alcoholic $CO_2$ and a quantity of $N/10$ HCl equal to the previously determined "Stage 2" value were aerated as described. For the 75 cc. mixtures, 5 minutes at room temperature or 3 minutes at 40–45° gave the correct titration value, and 2 minutes at 45° practically sufficed. Three minutes at 40–45° was also practically sufficient for the 130 cc. quantities but 5 minutes at room temperature was not quite long enough. 30 cc. quantities of alcoholic liquid made from the "alcohol extract" of the culture referred to on page 225, exactly alike in content of "extract" and HCl were aerated 3, 6, and 10 minutes at room temperature. The final titration results were identical.

It was evident that the rapidity with which the $CO_2$ was expelled depended largely upon the depth of the alcoholic liquid. The deeper the liquid the slower the expulsion. Possibly some part of this effect was due to the fact that when the smaller volumes were aerated there was more room for the splashes and more vigorous air currents could be used without fear of contaminating the outlet tube. Consequently, using the 250 cc. Erlenmeyer flask (p. 223) it seems advisable to limit the volume of alcoholic liquid actually aerated to about 50 cc. for room temperature estimations, and to aim at volumes nearer 30–35 cc. with a "Stage 2" value of 2 to 5 cc. $N/10$ solution.

*Possible explanations.* Traces of $CO_2$ dissolved in distilled water are removed with difficulty even on boiling. Apparently the rate at which the $CO_2$ is expelled from water depends mainly upon the effects of temperature and dilution upon the molecular and electrolytic dissociations of the carbonic acid. The greater the mass of the water relative to the quantity of carbonic acid present, the greater the depression of the dissociation: $H_2CO_3 \rightleftharpoons H_2O + CO_2$. Consequently the $CO_2$ is expelled at a slower rate as the dilution increases through $CO_2$ expulsion. Neglecting the possibility that the dissociation constant increases with rising temperature, the electrolytic dissociation would increase with increasing dilution, and it seems probable that the corresponding decrease in the concentration of un-ionised $H_2CO_3$ might produce a further adverse effect upon the molecular dissociation especially after boiling for some time, when the dilution would be extremely high. Perhaps the addition of other acids facilitates the complete expulsion of $CO_2$ owing to the increase in concentration of un-ionised $H_2CO_3$ caused by their H ions depressing the electrolytic dissociation.

The addition of alcohol to aqueous carbonic acid solutions virtually brings about the removal of water, thus tending to eliminate the adverse effects mentioned.

I have not made a systematic study of the rates at which $CO_2$ may be

expelled from aqueous solutions under varying conditions of temperature and dilution. Actual boiling is always regarded as essential, however, in those analytical processes which depend upon the complete expulsion of $CO_2$ after adding excess of mineral acid. In the case of one such process, namely that of Amos [1906] for estimating carbonates in the soil when the content is small, it is now generally recognised that the hot aqueous mineral acid exercises a decomposing effect upon the soil's organic matter to yield appreciable amounts of $CO_2$. Therefore when organic matter is present application of heat is very undesirable.

It will be shown in another communication that the dissociation constants of "weak" nitrogenous bases seem to be lowered to about one-thousandth of the values exhibited in water on adding alcohol up to 87 % by volume to the aqueous solutions, their dissociation curves then occupying correspondingly higher positions in the $[H^+]$ scale. Apparently in 87 % alcohol their titration curves commence, whereas in water they finish, at about $p_H$ 8·5. Hence alcohol added up to 87 % to aqueous solutions of salts of such bases would liberate the acids to an extent depending upon the resulting $[H^+]$ which would be determined by the "strength" of the acids. It appears therefore that the $CO_2$ was expelled from the "alcohol extract" of the culture which contained its $CO_2$ as bicarbonates of such bases (p. 225) practically as easily when no HCl had been added owing to the fact that it was virtually a solution of "free" carbonic acid which was aerated. Probably no loss of volatile bases occurred during this aeration because the organic acid radicles possessed the power of keeping the $[H^+]$ at a high enough level under the room temperature conditions.

## Summary.

Strong alcohol in which carbonic acid and small quantities of "free" volatile acids are dissolved very readily parts with the whole of the $CO_2$ but completely retains the volatile acids on aeration. In this solvent, bicarbonates of "weak" nitrogenous bases and "free" carbonic acid lose their $CO_2$ on aeration with equal readiness. A simple rapid room temperature process and a useful modification, applicable to fluids such as bacterial cultures, for estimating and eliminating the carbonic acid radicle, are based upon these observations. There is no risk of loss or change in any of the other constituents and subsequent determinations are more precise.

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# XXXII. THE ANTICOAGULANT ACTION OF PEPTONE *IN VITRO*.

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(*Received December 21st, 1927.*)

The effect of commercial peptone in causing delay of coagulation of blood *in vitro* has been investigated by Schmidt-Mulheim [1880], Afanassiew [1884], Pollitzer [1886], Halliburton [1888], Camis and Gley [1896], Arthus [1920], and Pickering and Hewitt [1922]. The opinion has been widely held that peptone has no specific action upon shed blood and can produce delay of coagulation or incoagulability of blood only *in vivo*, but the last two observers showed that peptone acts directly upon the constituents of the blood, without, however, determining to which of the constituents this action is directed.

The problem becomes relatively simple if the coagulation of fibrinogen by thrombin is studied in the presence of sodium citrate: under these conditions of experiment it may be assumed that the action of peptone is directed either to fibrinogen or to thrombin.

This action is found to be rapid compared with the process of coagulation. No indication is afforded by comparing the effect of mixing peptone for some time with thrombin before adding fibrinogen, or with fibrinogen before adding thrombin: the time of appearance of coagulation is found to be the same in both cases.

In the course of a search for possible methods of investigation it appeared likely that an indication of the nature of the anticoagulant action of peptone would be revealed by comparison with the effect of dilution of fibrinogen in presence of varying concentrations of thrombin, this being exhibited by curves (Fig. 1), each of which represents the coagulation periods of a series of dilutions of fibrinogen (citrated human plasma) to which a fixed amount of thrombin[1] has been added. Although this expectation was not fulfilled, as will be seen later, it may, nevertheless, be of interest to describe the line of enquiry followed since it reveals the limitations of a method which seemed at first likely to be fruitful of result.

The experiments made are recorded in Table I. They were carried out at room temperature (16° to 18°). In each experiment five parts of plasma (undiluted or diluted as indicated in column 2) were mixed with one part of

[1] As source of thrombin the venom of *Echis carinatus* [cp. Barratt, 1913] was used. I am indebted to Sir Charles Martin, F.R.S., Director of the Lister Institute, for kindly providing me with a supply of this venom.

citrated 0·85 % sodium chloride solution containing thrombin or a mixture of thrombin and peptone (columns 3 and 4).

Table I.

| Experiment | | Concentration of plasma employed % | Amount of thrombin (g.) in 1 cc. | Amount of peptone (g.) in 1 cc. | Onset of coagulation min. | Fibrils visible min. |
|---|---|---|---|---|---|---|
| A. | 1. | 100 | $167 \times 10^{-6}$ | — | 0·8 | 1 |
| | 2. | 30 | ,, | — | 1·2 | none |
| | 3. | 10 | ,, | — | remained liquid | ,, |
| | 4. | 5 | ,, | — | ,, | ,, |
| | 5. | 2·5 | ,, | — | ,, | ,, |
| B. | 6. | 100 | $10{\cdot}2 \times 10^{-6}$ | — | 2·7 | 3·2 |
| | 7. | 80 | ,, | — | 3·7 | 5·5 |
| | 8. | 60 | ,, | — | 5·5 | 7 |
| | 9. | 40 | ,, | — | 6 | none |
| | 10. | 30 | ,, | — | 9 | ,, |
| | 11. | 20 | ,, | — | 9 | ,, |
| | 12. | 10 | ,, | — | 9 | ,, |
| | 13. | 5 | ,, | — | remained liquid | ,, |
| | 14. | 2·5 | ,, | — | ,, | ,, |
| C. | 15. | 90 | $1{\cdot}50 \times 10^{-6}$ | — | 13 | 13 |
| | 16. | 80 | ,, | — | 18 | 18 |
| | 17. | 70 | ,, | — | 18 | 18 |
| | 18. | 50 | ,, | — | 25 | 25 |
| | 19. | 20 | ,, | — | 48 | 51 |
| D. | 20. | 100 | $0{\cdot}635 \times 10^{-6}$ | — | 19 | 19 |
| | 21. | 30 | ,, | — | 120 | 300 |
| E. | 22. | 100 | $10{\cdot}2 \times 10^{-6}$ | 0·02 | 22 | 34 |
| | 23. | 80 | ,, | 0·02 | 52 | 60 |
| | 24. | 60 | ,, | 0·02 | 55 | 90 |
| | 25. | 40 | ,, | 0·02 | 140 | 300 |
| | 26. | 30 | ,, | 0·02 | remained liquid | none |

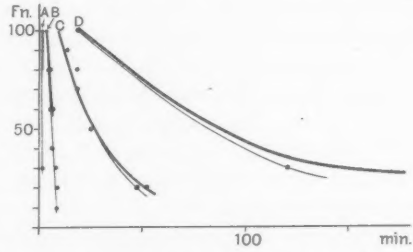


Fig. 1. The ordinates represent percentage of citrated plasma employed: the abscissae periods of coagulation in minutes. The thin portions of the curves correspond to "setting," the thick portions to the appearance of fibrin fibrils. In the two curves to the left (A, B) it will be observed that "setting" is still recognisable when fibrils have ceased to be visible.

The first twenty-one experiments, arranged in four series, afford data for the four curves shown in Fig. 1. For the first two series, Exps. 1–5 and 6–14, a relatively high concentration of thrombin was employed. In these series coagulation (indicated by fine lines on the figures) ceased to be recognisable when the dilution of plasma (Fig. 1) used was below 30 % and 10 % respectively: before this occurred the appearance of fibrin fibrils (indicated by

thickened lines), which were observed to be numerous, short and fine, had already ceased. In the third series, Exps. 15–19, a curve (C) of increased obliquity is obtained. The last two experiments, 20–21, lie on the curve (D) approaching still more nearly to the horizontal shown on the extreme right of Fig. 1: here, relatively little thrombin was added and the fibrils formed were larger, thicker and less numerous than in the preceding series, "setting" being feeble.

To the above twenty-one experiments giving four normal dilution curves a further series, Exps. 22–26, was now added. In these the conditions of experiment were similar (Fig. 2) to those obtaining in Exps. 6–14, except that 2 % of peptone was present: coagulation was retarded in each of the first four experiments and was absent in the last.

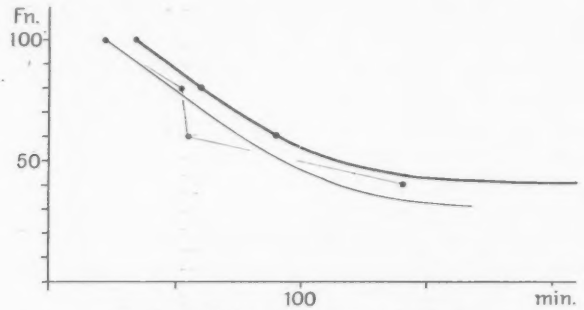


Fig. 2. Ordinates and abscissae as in Fig. 1.

If the action of peptone is upon fibrinogen and is comparable in its effect to dilution of fibrinogen solution (plasma) the coagulation periods in Exps. 22–26 would lie upon the corresponding curve (B) in Fig. 1, it being assumed that this curve could be produced so as to pass to the right close to the axis of abscissae. But actual experiment shows that in dilution experiments the curve cannot be produced so far to the right, both coagulation and the formation of visible fibrin fibrils ceasing while it is still near the axis of ordinates, as exhibited in Fig. 1. This difference would appear to indicate that the action of peptone is not directed to fibrinogen.

If, on the other hand, the action of peptone is exerted upon thrombin causing diminution of the amount of free thrombin present, then the coagulation periods could be plotted against the corresponding dilutions of fibrinogen as in Fig. 1. When this is done the curve shown in Fig. 2 is obtained, which assumes fairly closely the type exhibited in Fig. 1, lying a little to the right of the curve (D) in this figure. The close correspondence of the two curves suggests that the action of peptone is directed to thrombin.

On the basis of these results the conclusion appeared to be indicated that peptone had no effect upon fibrinogen, but entered into some kind of reaction with thrombin. Later investigations, however, necessitated a reconsideration

of this interpretation. In particular it became evident that the tacit assumption made above that the effect of peptone, if directed to fibrinogen and thereby causing a diminution in the amount of fibrinogen present, would be in all respects, that is to say quantitatively as well as qualitatively, identical with that of dilution of fibrinogen, is not necessarily valid when fibrinogen is regarded as a colloid. If the disappearance of fibrinogen could be effected without dilution longer coagulation periods might conceivably be obtained than is possible in dilution experiments.

In this dilemma attention was turned to another means of testing whether the action of an anticoagulant is exerted upon fibrinogen or thrombin, namely by determining the effect on fibril formation. Several attempts had already been made unsuccessfully to ascertain the nature of the action of peptone in this way. Additional experiments, now undertaken, were attended with success and enabled the conditions to be defined under which the action of peptone on fibril formation could be exhibited.

Before proceeding further the factors which normally determine the characters of fibril formation when coagulation occurs must be considered. When thrombin is added to fibrinogen (plasma) in diminishing amounts the fibrils appearing become reduced in number, but increased in length and thickness: on the other hand, when the amount of fibrinogen is diminished (by dilution), the quantity of thrombin added being unaltered, the fibrils formed do not increase in length and thickness, but may be observed to become definitely shorter and finer, this being attended, as in the former case, by a reduction in number and final disappearance [Barratt, 1921]. Both types of change are exhibited in Exps. 1–21: the former, for example, is seen when Exp. 6, in which the fibrils formed were numerous, $5\mu$ to $15\mu$ long and $0{\cdot}2\mu$ to $0{\cdot}3\mu$ thick, is compared with Exp. 20, in which the fibrils were less numerous, $40\mu$ to $100\mu$ long and $0{\cdot}3\mu$ to $0{\cdot}5\mu$ thick; the latter is observed in each of the four series.

When peptone was employed in Exps. 22–26 the effect upon the characters of the fibrils appearing was equivocal: some increase in length and thickness was observed in most experiments, but the degree of increase was less than that noted in corresponding experiments with nearly the same coagulation period in the normal series 1–21 and was not sufficiently marked to be completely outside the variations normally met with. It was in consequence of the failure to obtain a decisive indication of the influence of peptone upon fibril formation that extended investigation with a series of dilutions of fibrinogen of the type exhibited by Figs. 1 and 2 above was entered upon.

The principal cause of the difficulty experienced in determining the effect of peptone upon fibril formation was found to be the feeble character of the anticoagulant action of the samples employed. If a sample possessing a sufficiently powerful anticoagulant action can be obtained, the effect of peptone on fibril formation is readily shown. The conditions of experiment require to be so arranged that, on the one hand, (1) coagulation occurs very rapidly,

*i.e.* within one minute or less, in the control experiments in which no peptone is added; while, on the other hand, (2) the delay of coagulation caused by peptone is extremely marked, extending to one or several hours without, however, causing complete disappearance of fibrils. Dilution of plasma should be, as far as possible, avoided.

The effect of peptone upon fibril formation, when these conditions are fulfilled, is shown in Table II, Exps. 27–28: the quantities (present in 1 cc.) of plasma, thrombin and peptone employed are given in columns 2, 3 and 4 respectively. In this table it will be seen that a coagulation period of 50 sec., when thrombin acted upon fibrinogen in the absence of peptone (Exp. 27), was changed by the addition of 4·5 % of peptone into one of 60 min. (Exp. 28). In the former experiment the fibrils seen were very numerous, short and fine: in the latter the length of the fibrils was not markedly changed, but their number was diminished and they were barely visible. With further addition of peptone the fibrils became amicroscopic and when the amount was increased to 7 % "setting" was no longer observed.

Table II.

| Experiment | Plasma (cc. per cc.) | Thrombin (g. per cc.) | Peptone (g. per cc.) | Onset of coagulation | Fibrils | | |
|---|---|---|---|---|---|---|---|
| | | | | | Number | Length | Thickness |
| 27. | 0·80 | $10·2 \times 10^{-6}$ | — | 50 sec. | very numerous | 3 $\mu$ to 15 $\mu$ | 0·1 $\mu$ to 0·2 $\mu$ |
| 28. | 0·80 | „ | 0·045 | 60 min. | moderately numerous, barely visible | 4 $\mu$ to 7 $\mu$ | 0·1 $\mu$ |
| 29. | 0·80 | $0·037 \times 10^{-6}$ | — | 77 min. | scanty | 50 $\mu$ to 200 $\mu$ | 0·5 $\mu$ to 0·9 $\mu$ |

The effect of addition of peptone, exhibited in Exps. 27–28, is thus identical, so far as concerns the appearance of fibrils, with that observed when fibrinogen (citrated human plasma) is diluted with citrated 0·85 % sodium chloride solution, as in Exps. 6–14, Table I: in the latter series the fibrils, though diminishing in number and becoming increasingly difficult to observe, remained throughout of nearly the same length, namely 5 $\mu$ to 15 $\mu$. These results indicate unequivocally that peptone acts upon fibrinogen, which, in consequence, becomes diminished in amount just as occurs upon dilution.

For the sake of comparison with the effect of diminution of the amount of thrombin added, Exp. 29 is included in Table II: here the period of coagulation approximates to that of Exp. 28, but the fibrils, much reduced in number, are very markedly increased in length and thickness.

Table II serves to illustrate the action of two classes of anticoagulants, delay of coagulation being attended in the one by increased length and thickness of the fibrils, in the other by an extreme degree of fineness of these structures, which become barely visible, and are usually reduced in length also: in both a marked diminution in number is observed when a relatively considerable

amount of the anticoagulant has been added. Of these two classes the first is exemplified by hirudin, the second, to which peptone belongs, by sodium hydroxide [Barratt, 1921, 1926]: the former acts upon thrombin, the latter on fibrinogen.

Reference may now be made again to the circumstance that when peptone is added to plasma, in presence of a relatively large amount of thrombin, much greater delay of coagulation can be produced than is the case when plasma is simply diluted. The same is observed when sodium hydroxide is used in place of peptone: in this respect the two anticoagulants are in complete agreement. A further point of similarity is in the formation of afibrillary clot: this is readily obtainable with sodium hydroxide [cp. Howell, 1916], less so with peptone, the use of which renders some degree of dilution unavoidable.

SUMMARY.

Experiments made to determine the effect of peptone upon fibril formation in citrated fibrinogen solution (blood-plasma) *in vitro* show that peptone (1) acts upon fibrinogen, which becomes in consequence diminished in amount, and (2) is without action upon thrombin.

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# XXXIII. CHEMICAL CHANGES IN MUSCLE.

## PART I. METHODS OF ANALYSIS.

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Some of the chemical changes responsible for activity in vertebrate skeletal muscle are fairly well known. On the other hand, in plain muscle, cardiac muscle and certain muscles of invertebrate animals, these changes are not clearly understood, possibly because the analytical methods which have been used for vertebrate skeletal muscle do not always give good results on application to other types of muscle. For this reason it was thought advisable to examine and if possible to improve the methods available for the estimation of carbohydrates and lactic acid in muscle.

### *Treatment of muscle preparatory to analysis.*

In order to follow the changes involved in muscular activity, the tissue must be fixed in a known state and lactic acid, glycogen and other carbohydrates estimated. Fletcher and Hopkins [1907] found that special treatment was necessary to avoid formation of lactic acid before analysis. Alcohol was used at a temperature below 5° to precipitate protein and extract lactic acid. They minced the muscle in the cold alcohol and then ground it with sand until extraction was complete. Laquer [1914] and Schenck [1924] used dilute hydrochloric acid at $-10°$ and subsequently precipitated interfering substances with mercuric chloride. Eggleton and Eggleton [1927] used cold trichloroacetic acid solution to extract soluble phosphate from muscle; this seems to be a good method for use when acid soluble substances alone are to be estimated. It would, however, be unsuitable if glycogen were to be estimated on the same sample of muscle. Freezing of the tissue with liquid air or carbon dioxide snow, followed by grinding, should give even sampling and prevent post-mortem lactic acid production, but Meyerhof [1926] found that resting values for lactic acid obtained by this method were not lower than those obtained by the use of cold alcohol. Inositol can be estimated, after the method of Needham [1923], in the residue, after extracting the muscle with alcohol. The inositol is extracted with acetone and the residue from this extraction can be used for glycogen estimation. When lactic acid and glycogen are to be estimated in

the same sample of muscle, alcohol must be used to extract the lactic acid and precipitate the glycogen and protein. For complete precipitation of the glycogen the alcohol should be stronger than 60 %, while the protein is better precipitated with 80 % alcohol.

*Method.* The muscle is minced with alcohol cooled in a freezing mixture to about $-5°$ and then made up to a known definite volume, five times the volume of the muscle. After standing overnight, the alcohol is filtered off and a measured volume of the filtrate evaporated for the estimation of lactic acid and lower carbohydrates. When the alcohol has been removed by evaporation, the extract must be cleared of lipins and protein. If present in large amounts, hexoses must also be removed. The extract is therefore treated with ammonium sulphate solution or phosphotungstic acid. Lactic acid can be separated from hexoses in an ammonium sulphate solution by extracting the acid with ether as did Fletcher and Hopkins, or with amyl alcohol as described by Ohlson [1917]. If the lactic acid is to be estimated by oxidation, however, it is advisable to avoid extraction with ether as this usually contains aldehydes which would probably affect the estimation of lactic acid. Hexoses may be removed from a phosphotungstic acid solution by precipitation with copper sulphate and calcium hydroxide, as used by Van Slyke [1917]. In many cases the amount of hexoses present is too small to have any marked effect on the lactic acid estimation. The error due to hexoses is small, as is shown in Table I, but it is too irregular for the application of a correction.

Table I. *The effect of glucose on the estimation of lactic acid, by the oxidative method, using steam distillation.*

| | | | | | |
|---|---|---|---|---|---|
| 5·0 mg. glucose required | 3·7 | 0·5 | 1·2 | 1·7 | 0·9 cc. *N*/1000 iodine |
| equivalent to | 0·17 | 0·02 | 0·05 | 0·08 | 0·04 mg. lactic acid |
| 10·0 mg. glucose required | 4·3 | 5·0 | 1·1 | 3·0 | 2·1 cc. *N*/1000 iodine |
| equivalent to | 0·19 | 0·22 | 0·05 | 0·13 | 0·09 mg. lactic acid |

The evaporated alcoholic extract is therefore heated with 10–20 cc. of saturated ammonium sulphate solution for half-an-hour on the water-bath. The last traces of alcohol are then removed by heating with 2 cc. of benzene, when the low boiling alcohol-benzene-water tertiary mixture quickly boils off. The precipitated fat and protein are filtered off through asbestos and washed with saturated ammonium sulphate solution. The filtrate and washings are made up to a known volume with saturated ammonium sulphate solution and aliquot parts taken for estimation of lower carbohydrates and lactic acid. The solution will not keep indefinitely, so that estimations should be made within two days.

*Estimation of total carbohydrates.*

In examining the changes occurring in muscle it would be convenient to estimate the total carbohydrates present, if possible, in one estimation. This would provide an excellent check on the separate estimation of glycogen and lower carbohydrates, but no simple method of determination of the total carbohydrates could be found. By hydrolysis of the whole tissue with acid,

all carbohydrates would form simple hexoses, while the protein would largely form amino-acids. The amount of protein degradation products, however, is too great to allow of estimation of the hexoses even by a ferricyanide method. Such products of hydrolysis give the biuret reaction; they also give large and irregular glucose titration values. After ultra-filtration through collodion, which should remove the protein compounds, the result is very little better. Attempts to separate the glucose with alkaline copper sulphate are useless, owing to the combination of the copper with the amino-compounds present. The carbohydrates must be estimated therefore in two parts—soluble and insoluble in alcohol—or lower carbohydrates and glycogen respectively. This has the disadvantage that some dextrins may not be estimated.

*Glycogen estimation.*

Glycogen is estimated by methods which are variations on the procedure described by Pflüger [1905]. Improvements in the method for estimation of small amounts have been made by Evans [1926].

*Method.* The residue of tissue insoluble in alcohol is heated for 3 hours on a water-bath with 0·25 cc. of 60 % potash for each gram of original tissue. When cool, the hydrolysed material is diluted with water to three times the volume of potash taken. Alcohol is added till the total volume is seven times the original volume of potash. After standing overnight, a precipitate of glycogen forms and is filtered off on asbestos on a Gooch filter. It is well washed with 60 % alcohol and the washed precipitate is transferred with the asbestos into a beaker. 50 to 100 cc. of 2·2 % hydrochloric acid are added and the beaker and contents heated for 5 hours on a boiling water-bath. The solution is then filtered and the filtrate and washings made up to a known volume. Aliquot parts of this are taken for estimation of glucose, which is effected by oxidation with excess of alkaline ferricyanide and determination of the excess iodometrically [Hagedorn and Jensen, 1923; Issekutz and Both, 1927]. For the estimation of an amount below 5 mg., the glucose is heated for 15 minutes in a flask in a boiling water-bath with 20 cc. of a solution containing 0·33 % potassium ferricyanide and 2·12 % sodium carbonate. When cooled, 10 cc. of a solution containing 7·5 % zinc sulphate, 25 % sodium chloride and 5 % potassium iodide, followed by 10 cc. of 3 % acetic acid, are added. The liberated iodine is titrated with $N/100$ sodium thiosulphate. A blank is carried out on 20·0 cc. of the ferricyanide solution without glucose. The difference in titre between blank and determination is almost proportional to the amount of glucose present, as shown in Table II.

Table II. *Estimation of pure glucose.*

| mg. glucose | Equivalent $Na_2S_2O_3$ in cc. | | | mg. glucose per cc. $Na_2S_2O_3$ |
|---|---|---|---|---|
| 1·25 | 3·90 | 3·95 | 3·90 | 0·315 |
| 2·50 | 8·00 | 7·90 | 7·95 | 0·32 |
| 3·75 | 12·0 | 12·1 | 12·0 | 0·31 |
| 5·00 | 16·1 | 16·1 | 16·0 | 0·31 |

The error of assuming the titre to be proportional to the glucose present, is thus negligible. The sodium thiosulphate is standardised by carrying out the estimation with a standard glucose solution preserved with benzoic acid. Holden [1926] showed this method of glucose estimation to be less sensitive to error from amino-acids than the copper reduction methods. It is also unaffected by ammonium sulphate solution, which makes it useful for estimating lower carbohydrates in such a solution. The estimation by this method, however, is impossible in the presence of moderate amounts of protein degradation products. After treating as in the glycogen estimation, muscle protein previously freed from glycogen reduces ferricyanide to some extent, as shown in Table III. Another error arises from the incomplete hydrolysis of glycogen by acid. Nerking [1901] obtained a 97 % yield of glucose after boiling for 3 to 5 hours. Harden and Young [1902] found that the rate of hydrolysis slowed down after 4 hours; they obtained maximum conversions of 96 % with rabbit glycogen after 8 hours, 97·6 % with yeast glycogen after 7 hours and 98·3 % with oyster glycogen after 7 hours at 100°. A sample of purified *Mytilus* glycogen, kindly supplied by Dr Slater, gave yields of about 93 % after 5 hours' hydrolysis, as shown in Table III. The average conversion of glycogen into glucose in presence of lobster or frog muscle is 92 %. It is necessary to allow for this in the calculation of results, which should be accurate to less than 5 %, using 10 g. of tissue containing at least 5 mg. of glycogen. It is convenient to state the results in terms of the hydrate $(C_6H_{12}O_6)_n$ which Slater [1924] has shown to exist.

Table III. *Glycogen estimations with purified* Mytilus *glycogen containing* 96·2 % $(C_6H_{12}O_6)_n$.

| Amount taken | mg. glucose | % recovery and conversion | |
|---|---|---|---|
| 5·00 mg. glycogen | 4·43 | 92·7 | |
| 5·00 mg. glycogen | 4·48 | 93·8 | |
| 2·0 g. dried lobster muscle | 0·95 | — | |
| 2·0 g. dried lobster muscle | 0·74 | — | |
| 2·0 g. dried lobster muscle and 5·00 mg. glycogen | 5·28 | 92·3 | Allowing for muscle blank |
| 2·0 g. dried lobster muscle and 5·00 mg. glycogen | 5·13 | 88·0 | Allowing for muscle blank |
| 4·72 g. frog muscle | 16·6 | — | |
| 4·72 g. frog muscle and 37·0 mg. glycogen | 50·8 | 92·6 | |
| 6·43 g. frog muscle | 25·4 | — | |
| 6·43 g. frog muscle and 37·0 mg. glycogen | 60·3 | 94·0 | |

*Estimation of lower carbohydrates.*

The so-called lower or soluble carbohydrates of muscle are probably intermediates in the conversion of glycogen into lactic acid. Although they do not change apparently to any great extent in muscular work, yet they have some significance. Usually quick moving muscles contain more than slow muscles; the muscles of summer frogs contain more than those of hibernating frogs.

Parnas and Wagner [1914] estimated the carbohydrate extracted with 60 % alcohol and boiling water. These two extracts were combined; they contained all the soluble carbohydrate and a little of the glycogen. Mercuric acetate and caustic soda were added to precipitate protein. Excess mercury was removed with hydrogen sulphide, which in turn was removed by blowing air through the solution. Hydrochloric acid was added to make it up to 2·0 % hydrochloric acid and it was heated for 2 hours on the water-bath, after which the glucose was estimated by the Bertrand method. Meyerhof [1920, 2] simplified this by extracting the muscle with 60 % alcohol only and estimating glycogen in the residue. The same methods of protein precipitation and hydrolysis were used. By use of the modified Hagedorn and Jensen method of glucose estimation, which is not influenced by ammonium salts, it is possible to estimate the lower carbohydrates in the same extract as is used for estimation of lactic acid.

A measured volume of the muscle extract which has been treated with ammonium sulphate and benzene, as previously described, is made up to 2 % hydrochloric acid. This is heated for 2 hours on the water-bath, neutralised, and the glucose present is estimated by heating with excess ferricyanide solution. In some instances, such as lobster muscle the heating with acid can be omitted without affecting the results; this is not so with frog muscle.

*Estimation of lactic acid.*

Although lactic acid can be estimated gravimetrically, colorimetrically, volumetrically and by purely physical methods, no method has the accuracy and reliability that is desired. Long [1924] and others have indicated that the only method suitable for the estimation of small amounts of lactic acid is the oxidative process. In this process the acid is oxidised to acetaldehyde which is determined iodometrically. If more than 50 mg. is to be estimated, isolation as zinc lactate [Fletcher and Hopkins, 1907] is usually adopted. Phelps and Palmer [1917] isolated and weighed quinine and guanidine lactates, which are insoluble in carbon tetrachloride. This method is rather tedious and appears to be little used. The main objection to the oxidative method is that it is not very specific and should only be used on pure solutions of lactic acid. On the other hand, the method of Fletcher and Hopkins is not free from this defect; it is, for example, interfered with by succinic acid and by urea, while the oxidative method is not. Moyle [1924] has shown that rabbit muscle may contain 0·1 % of succinic acid and this would cause lactic acid values determined by the zinc lactate method to be too high. Succinic acid is soluble in ether and zinc succinate is soluble in water, though it contains 44·1 % zinc oxide, as compared with 33·4 % for zinc lactate. It is difficult to distinguish between crystals of pure zinc lactate and of zinc lactate contaminated with zinc succinate, from the appearance of the crystals. The resting value of lactic acid, 0·02 % obtained by Fletcher and Hopkins is probably too high for this reason. Meyerhof [1920, 1] using the oxidative method of estimation obtained a lower resting value of 0·01 %. Fletcher and Hopkins do not give any analyses of

zinc content of zinc lactate obtained from resting muscle, probably on account of the small amount of zinc lactate obtained in those experiments. The lower figure obtained by the oxidative method is almost certainly nearer to the true resting value than that originally obtained. The determinations of lactic acid in fatigued muscle and in muscle in rigor will be less affected than the determinations of the small amount of lactic acid found in resting muscle. On account of the unspecific nature of the methods of estimation, lactic acid should be identified qualitatively before any quantitative method is applied. It is then advisable to estimate the acid by at least two methods and to compare the results obtained.

Long [1924] has examined the early work on the oxidative method. He adopted with slight modification the method published by Clausen [1922]. The oxidation of the lactic acid, by sulphuric acid or potassium permanganate, gave acetaldehyde which was collected in sodium bisulphite solution. After oxidising the excess bisulphite with iodine, the aldehyde-bisulphite compound was decomposed with sodium bicarbonate and the liberated bisulphite titrated with standard iodine. This accurate method of estimating acetaldehyde, due to Clausen, made the estimation of lactic acid much more reliable than it had ever been before. Since Clausen's paper, the only improvements have resulted in the increase of the yield from 85 to 98 % and reduction of the time necessary to make the estimation. Long obtained better oxidation with potassium permanganate than with sulphuric acid. Brehme and Brahdy [1926] used sulphuric acid. They heated the lactic acid with sulphuric acid, for 1 to 1½ hours, at 140° in an all glass apparatus in a metal-bath. They obtained yields of 92 to 95 %, but their process was very lengthy. Friedeman, Cotonio and Shaffer [1927] describe a modification of the Clausen process, by means of which estimations take only 15 minutes and a 96 % yield is obtained. The improvements lie in the apparatus for carrying out the oxidation and in the use of manganese sulphate as catalyst. This addition of manganese sulphate may be necessary with pure lactic acid, as without this there is often a considerable induction period before oxidation begins; manganese sulphate is then formed and the action proceeds rapidly. In estimating lactic acid from any biological source there is practically no induction period, as traces of organic impurity are first oxidised, with simultaneous production of manganese sulphate.

In the above methods with the exception of those of Meyerhof [1920, 1] and Long [1924], an air current is used to carry the acetaldehyde from the oxidising vessel to the absorption vessels. Meyerhof and Long used direct distillation. The air current, which is obtained by sucking air through the apparatus, will contain the variable impurities of the laboratory air. Alcohol or acetone vapour in the air would materially affect the results. In order to avoid this source of error, Brehme and Brahdy found it necessary to use outside air, led by a tube from outside, to the apparatus; this however is cumbersome. In addition to this, the Consortium für elektrochemische Industrie [1913] state

that manganese sulphate is an excellent catalyst for the aerobic oxidation of acetaldehyde to acetic acid. It would therefore seem advisable to avoid the presence of air, if possible, in the estimation.

Some experiments were made using direct distillation in the determination, after the method of Meyerhof, but this did not give good results, as can be seen from Table IV. This is probably due to over-oxidation of the acetaldehyde to acetic acid, owing to insufficient agitation allowing acetaldehyde to remain too long in the presence of the permanganate. Steam distillation should avoid this and on trial gave better results. At first this was performed in a Claisen flask fitted with rubber stoppers. Later the apparatus[1] (Fig. 1) was designed for

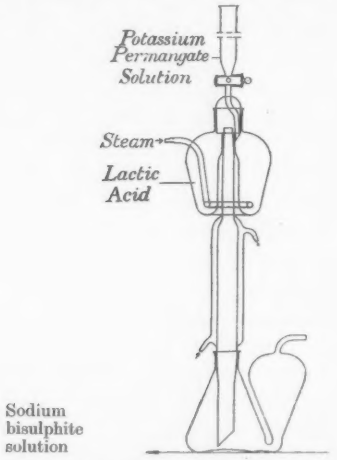


Fig. 1. Apparatus for the estimation of lactic acid.

the process; it gives slightly better results and is very convenient in working. This method gives the same results as that of Friedeman, Cotonio and Shaffer in a laboratory free from organic vapours. The method of steam distillation is independent of the air and generally gives more regular conditions for oxidation. Results obtained by various methods and the recovery of lactic acid from muscle are given in Table IV. Except for the use of steam and the standardisation of the iodine solution with a lactic acid solution, the procedure in the estimation is similar to that of Clausen.

Material containing between 0·5 and 5 mg. of lactic acid is placed in the upper vessel, with 10 cc. of 10 % sulphuric acid. 20 cc. of $N/20$ sodium bisulphite are placed in the Volhard flask. The condenser is well cooled with a fast stream of water and a vigorous current of steam is led through the solution of lactic acid. $N/200$ potassium permanganate is slowly dropped in from the funnel, so as to maintain a pink colour. The acetaldehyde produced by oxidation is immediately carried over by the steam, the distillate collects in the

[1] Made by the Scientific Glass Blowing Co., Manchester.

Table IV. *Estimation of* 1·00 *mg. lactic acid—from* 1·07 *mg. pure lithium lactate.*

| Process | cc. $N$/1000 iodine | mg. lactic acid | % yield average |
|---|---|---|---|
| Clausen | 20·8 | 0·94 | |
| | 18·2 | 0·83 | |
| | 15·9 | 0·72 | 85·2 |
| | 21·8 | 0·98 | |
| | 20·1 | 0·90 | |
| | 17·3 | 0·78 | |
| Friedeman, Cotonio and Shaffer | 21·2 | 0·95 | |
| | 20·9 | 0·94 | |
| | 20·1 | 0·90 | 93·6 |
| | 19·6 | 0·88 | |
| | 19·7 | 0·89 | |
| | 22·8 | 1·04 | |
| Direct distillation | 15·0 | 0·68 | |
| | 17·3 | 0·78 | |
| | 19·6 | 0·89 | 81·7 |
| | 18·5 | 0·83 | |
| | 19·1 | 0·88 | |
| | 18·4 | 0·84 | |
| Steam distillation | 20·9 | 0·94 | |
| | 19·8 | 0·89 | |
| | 20·6 | 0·93 | 92·1 |
| | 21·5 | 0·97 | |
| | 19·7 | 0·89 | |
| | 20·4 | 0·92 | |

Recovery of lactic acid from muscle.

| Muscle taken | mg. lactic acid | Recovery % |
|---|---|---|
| 4·73 g. frog muscle | 6·0 | |
| 4·73 g. frog muscle and 5·0 mg. lactic acid | 10·8 | 96 |
| 6·43 g. frog muscle | 20·2 | |
| 6·43 g. frog muscle and 5·0 mg. lactic acid | 25·4 | 104 |
| 3·97 g. frog muscle | 6·9 | |
| 3·97 g. frog muscle and 5·0 mg. lactic acid | 11·5 | 92 |
| Average | — | 97 |

bisulphite solution. In a few minutes the pink colour becomes permanent without further addition of permanganate and after another 5 minutes' distillation the steam is shut off and the apparatus dismantled. The condenser is washed down into the bisulphite in the Volhard flask. Starch is added to the bisulphite and the excess is titrated with $N$/20 iodine, to a pale blue colour. Saturated sodium bicarbonate solution is added to decompose the aldehyde-bisulphite compound and the bisulphite so liberated is titrated with $N$/1000 iodine solution. Each cc. of iodine should represent 0·045 mg. of lactic acid. The iodine is best standardised by carrying out the determination on a solution of pure lithium lactate. At least one standardisation should be made with each batch of estimations.

SUMMARY.

The methods of estimation of glycogen, lower carbohydrates and lactic acid have been examined. An improved method for the determination of lactic acid using steam distillation combined with oxidation is described.

I have great pleasure in thanking Mr A. D. Ritchie for help and advice, and Prof. H. S. Raper for continued interest and helpful criticism.

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# XXXIV. THE EFFECTS OF PHLORIDZIN AND OTHER SUBSTANCES ON FERMENTATIONS BY YEAST.

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(*Received December 23rd, 1927.*)

## Introduction.

This paper deals with a systematic investigation of the effects of a number of glucosides and polyhydric phenols on yeast fermentations.

The experiments to be described were originally undertaken to ascertain whether certain unsaturated substances such as allyl alcohol and acrylic acid, which have powerful effects on bacteria [Quastel and Wooldridge, 1927], have any action on the fermentative activity of yeast. By making use of an apparatus which is capable of yielding very accurate and reproducible results it was found that both these unsaturated compounds bring about an almost complete cessation of the fermentation of glucose by yeast and zymin. A number of other substances were then tested, among them a series of glucosides. Of the glucosides tried one only, phloridzin, showed a marked retarding action on the fermentation of glucose by zymin. Out of a series of polyhydric phenols, phloroglucinol was the most effective in retarding the rate of fermentation by zymin. It is significant that phloroglucinol is the phenol present in phloridzin. Phloridzin, however, had no perceptible action on the fermentation of pyruvic acid by zymin or by live yeast or on the fermentation of glucose by live yeast.

## The apparatus.

The apparatus used by us was originally designed by Tryhorn and Jessop in order to measure the rate of decomposition of hydrogen peroxide. It is suitable and extremely convenient for measuring the rate of any reaction involving the evolution of a gas, and also is more accurate than most pieces of apparatus used for the same purpose. As no full description of the device was published by Tryhorn and Jessop [1925] it will be given here. Essentially, it consists of a reaction vessel *A* (Fig. 1) connected through a three-way tap *B* (Fig. 2)[1] with two small gas burettes ($C_1$), each of which can be opened to the atmosphere by a simple tap ($D_1$). As will be seen in Fig. 3 the mounting of the apparatus is such as to enable the whole to be immersed in the water of a constant temperature bath. The reaction vessel (Fig. 1) used

[1] Only half of the apparatus is shown, the two burettes being duplicates (see Fig. 3).

has a capacity of 125 cc. At the bottom of the vessel is a drain tube, and through the top is led a stirring rod $F$, the outlet being closed by a mercury seal $M$. A side-tube, $G$, leads to the three-way tap $B$ (Fig. 2), and the gas burettes. The third outlet is a broad tube $H$, leading from the side of the vessel near the top, sufficiently long to project above the surface of the water of the thermostat. It is stoppered by inserting into the constriction at its base a glass rod of suitable diameter covered with tightly fitting rubber tubing, as this arrangement decreases the effective volume of the apparatus and therefore increases its accuracy. For the same reason the connecting tubes between the reaction vessel and the burettes are all capillaries. Independently attached to the base is a vertical iron rod holding a long bearing in which runs an axle carrying a small brass wheel. The axle is bored to an internal diameter such that the top of the stirring rod is held in it by friction. It is essential for the

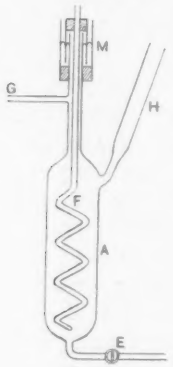


Fig. 1.

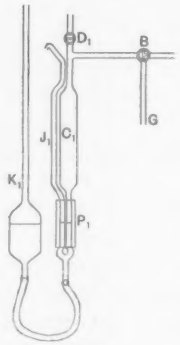


Fig. 2.

success of the apparatus that this axle be held in a long bearing to prevent the stirring rod wobbling and breaking the reaction vessel.

Each gas burette is of 5 cc. capacity and is connected at the lower end with a tube ($J_1$) which is of the same diameter as the narrow end tubes of the burette itself. This tube is open to the atmosphere and enables one to bring the gas within the apparatus to atmospheric pressure with great accuracy on filling or emptying the burettes. The mounting of the burettes is obvious from Fig. 3. Each burette has a zero graduation on the tube just above the bulb and a millimetre glass scale, $P_1$, is fastened to the tube below the bulb. The space between the tubes and scale is filled with Canada balsam to facilitate reading the scale. In order to be able to replace the scale in its original position, if it should be moved, the burette tube has a mark etched on it, to which the zero mark on the scale can be adjusted.

As manometric liquid one hundredth normal sulphuric acid saturated with carbon dioxide was used throughout and was found to be quite satisfactory.

No appreciable loss of gas can occur by diffusion through the long tubes of the manometer.

The burettes were calibrated in the usual manner [Treadwell, 1919] between the zero mark and each of three different graduations on the scale. A graph was constructed showing the scale reading corresponding to 5 cc. of gas at N.T.P. at different atmospheric pressures, correction being applied for the vapour pressure of water at 27°.

In all experiments the stirring rod was run at a speed of about 600 revolutions per minute from the thermostat stirrer. This speed is at least four times

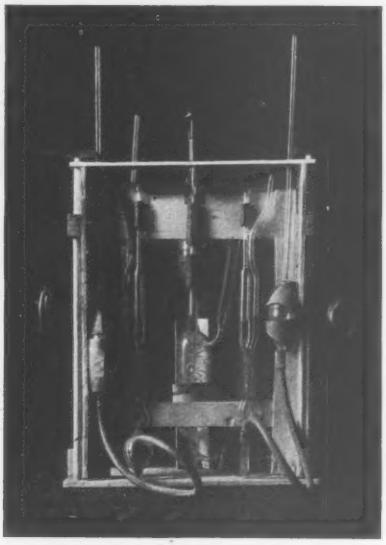

Fig. 3.

that necessary to ensure a constant supersaturation of gas in the zymin suspensions used. The thermostat is of such a width that the counterpoises of the manometers hang freely outside it; in all experiments it was kept at 27° by means of a heating bulb controlled by a toluene-mercury thermometer and electrical relay.

Before each experiment the reaction vessel is cleaned by filling with chromic acid cleaning mixture and allowing to stand for at least half an hour; and after washing it out with water the apparatus is placed in the thermostat and allowed two or three minutes to attain a steady temperature. When the reactants have been introduced into the vessel the latter is connected with one of the burettes by means of the three-way tap, and with the tap *D* open the manometer bulb is raised until the meniscus is at the zero mark. After closing the

neck by the stopper the tap *D* is closed and readings commenced. As carbon dioxide is evolved the manometer bulb is lowered so as to keep the pressure inside the apparatus approximately atmospheric until the meniscus reaches the burette scale, when the bulb is lowered until the meniscus in the tube *J* is at the mark corresponding to a corrected volume of 5 cc. When the meniscus in the burette tube has fallen to the same level the three-way tap is quickly turned to connect the reaction vessel with the other burette, which has already been set to zero and disconnected from the atmosphere. The exact time of turning the three-way tap is noted. By proceeding in this manner a series of readings of times and volumes is obtained. Care must be taken that the three-way tap is only turned when the pressure inside the apparatus is equal to that outside.

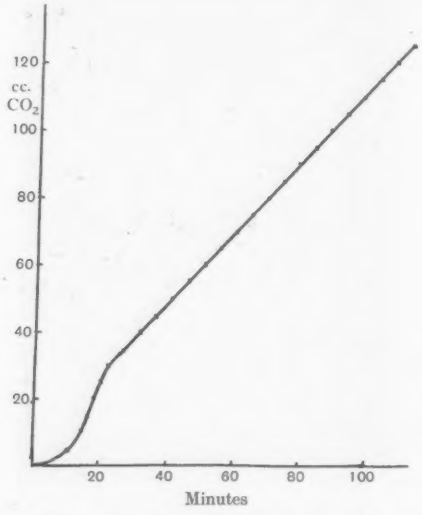


Fig. 4. Fermentation of glucose by zymin.

As 1 mm. on the scale corresponds to a volume of about $\frac{1}{100}$ cc. the error of reading the volume is less than $\frac{1}{10}$ %.

The greatest error in measuring the time taken to produce 5 cc. $CO_2$ (*i.e.* to fill each burette) lies in the interval required to note the time at which the 5 cc. mark of one burette is reached, for immediately this is reached a three-way tap must be turned so that gas flows into the second burette. This error may be appreciable with a reaction proceeding very quickly, but if 5 cc. $CO_2$ are collected in not less than 2 minutes the error is less than 1 %.

A typical curve of the fermentation of glucose by zymin (yeast treated with acetone) is shown in Fig. 4. The curve consists of two distinct parts, (*a*) an initial stage, lasting about 20 minutes, in which the velocity of $CO_2$ formation is at first slow and then rapid, and (*b*) a stage in which the production of $CO_2$

proceeds at a steady linear rate for at least 8 hours. The linear part of the curve, if produced, cuts the ordinate (in our experiments) at a point corresponding to about 7 cc. $CO_2$. The value of the steady rate is not changed by decreasing the rate of stirring to a fifth of its usual value. The linearity of the second part of the curve is indicated in Fig. 4, where there has been no smoothing of the curve, and in Table I. This table gives results carried out with two specimens of zymin, F and H, and demonstrates the constancy of the quotient $CO_2/t$, where $CO_2$ represents the volume of $CO_2$ formed in time $t$ when the steady state has been reached (*i.e.* after 20 minutes from the commencement of the fermentation). These results are typical for zymin.

Table I. *Rates of fermentation of glucose by zymin. Times taken after evolution of the first 30 cc. $CO_2$.*

| Zymin F | | | Zymin H | | |
|---|---|---|---|---|---|
| $t$ in min. | $CO_2$ | $CO_2/t$ | $t$ in min. | $CO_2$ | $CO_2/t$ |
| 10·4 | 10 | 0·96 | 9 | 10 | 1·11 |
| 21·1 | 20 | 0·95 | 19·25 | 20 | 1·04 |
| 62·5 | 60 | 0·96 | 57 | 60 | 1·05 |
| — | — | — | 96·2 | 100 | 1·04 |
| 113·25 | 110 | 0·97 | — | — | — |

With live yeast the second stage in the fermentation of glucose is not so markedly linear as with acetone yeast. The quotient $CO_2/t$ increases gradually (see Table II).

Table II. *Rate of fermentation of glucose by yeast. Times taken after evolution of the first 30 cc. $CO_2$.*

| $t$ | $CO_2$ | $CO_2/t$ |
|---|---|---|
| 7·15 | 10 | 1·40 |
| 13·9 | 20 | 1·43 |
| 20·5 | 30 | 1·46 |
| 26·9 | 40 | 1·48 |
| 33·3 | 50 | 1·50 |
| 39·65 | 60 | 1·51 |
| 45·5 | 70 | 1·54 |

### Preparation of zymin.

A Dutch baker's yeast was used for the whole of our experimental work. The yeast before use was stored in the ice chest. For preparation of the zymin the following procedure was adopted. The yeast was broken up into small lumps or crumbs and left on the bench at room temperature for 24 hours. 100 g. yeast were then stirred into 600 cc. acetone. After stirring well for 10 minutes the acetone was filtered off in a Büchner funnel and the yeast pressed very hard and powdered. The powder was then ground thoroughly for 2 minutes with 200 cc. acetone, filtered and pressed hard. Finally it was ground with 50 cc. ether for 3 minutes, filtered, pressed hard and dried on the Büchner funnel. The powder was spread on filter paper for half an hour at room temperature and then allowed to stand in the hot room (37°) for 24 hours.

The preparation was stored in the ice chest. Experience has shown that the best results are obtained when the yeast has been well pressed and powdered at each stage of the dehydration. If the final preparation appears granular or crumbly and not a fine powder its activity with glucose is remarkably poor. The yield of zymin from 1 kg. of yeast was 240 g.

### Reproducibility of results with a given specimen of zymin.

Our method of ascertaining the effects of substances upon the fermentation of glucose consists in adding the substance in question to the reaction vessel when the steady rate of $CO_2$ production has been progressing for 20 or 30 minutes. The rates of $CO_2$ evolution before and after the addition are compared. It is not essential therefore that the same specimen of zymin, or zymin of the same activity, should be used in every experiment. A number of experiments has shown that the same percentage inhibition of the rate of fermentation of glucose is produced by the addition of a given quantity of the inhibitor, when the initial steady rate before addition differs in different experiments.

That a given specimen of zymin, however, retains its activity intact for a considerable period is shown in Table III. This table gives results with zymin F, a number of different experiments being carried out with it on each of several days. No particular effort was made to ensure the reproduction of the same value of the steady rate of fermentation in each experiment, and yet it will be observed that these rates do not differ markedly from each other. The table also gives results with zymin M, a more active preparation than zymin F. In this case out of nine different experiments carried out within 4 days the times for the production of 80 cc. $CO_2$ lie, with one exception, within $\pm 1\frac{1}{4}$ % of the mean value.

### Influence of phloridzin, etc. on the fermentation of glucose by zymin.

*Experimental method.* For a reasonably fast fermentation a suspension of 8 g. zymin in 40 cc. 10 % glucose solution was used. 30 cc. water containing 4 g. glucose were placed in a conical flask which was placed in the thermostat (27°) until the solution had attained the temperature of the latter. 8 g. zymin were added to the solution, the flask was stoppered and well-shaken, and its contents were poured into the reaction vessel of the apparatus. The flask was washed with 10 cc. water, the washings being poured into the vessel. The latter was stoppered, stirring was commenced, and readings of the time required for the evolution of consecutive volumes of 5 cc. $CO_2$ were taken. No buffer was used, this being unnecessary, as shown by control experiments. For the exact comparison of the initial steady rates of fermentation in a series of experiments, it is important that the interval of time from the mixing of the zymin with the glucose to the commencement of gas collection should be constant,

and that the glucose solution should be immersed in the thermostat for the same period.

Table III. *Times taken after attainment of steady rate of fermentation.*

| Zymin F | | | Zymin M | | |
|---|---|---|---|---|---|
| Date | Exp. | Time in min. required to produce 20 cc. $CO_2$ | Date | Exp. | Time in min. required to produce 80 cc. $CO_2$ |
| June 14 | 1 | 20·0 | Nov. 24 | 1 | 34·8 |
| „ 14 | 2 | 21·6 | „ 24 | 2 | 35·2 |
| „ 15 | 3 | 23·0 | „ 24 | 3 | 34·4 |
| „ 16 | 4 | 22·2 | „ 25 | 4 | 35·1 |
| „ 16 | 5 | 22·4 | „ 25 | 5 | 36·5 |
| „ 16 | 6 | 21·6 | „ 27 | 6 | 35·2 |
| „ 17 | 7 | 22·0 | „ 28 | 7 | 35·2 |
| „ 17 | 8 | 22·0 | „ 28 | 8 | 34·7 |
| „ 20 | 9 | 23·0 | „ 28 | 9 | 34·8 |
| „ 20 | 10 | 23·0 | | | |
| „ 21 | 11 | 23·2 | | | |
| „ 21 | 12 | 24·0 | | | |

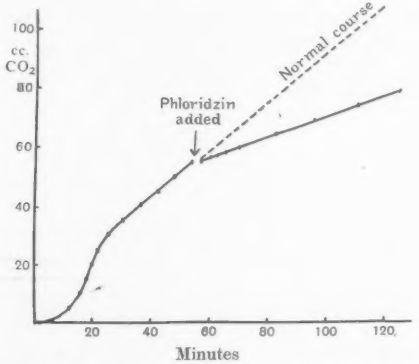


Fig. 5. Effect of phloridzin on zymin fermentation of glucose.

When the rate of $CO_2$ evolution had become steady for 20 or 30 minutes the reaction vessel was unstoppered, the substance under investigation added, the vessel restoppered and readings resumed. The substance added was usually either dissolved or suspended in water. The time lost by this process of addition was usually less than a minute.

### Typical effect of phloridzin.

A typical curve illustrating the effect of addition of phloridzin to a glucose fermentation by zymin is shown in Fig. 5. At the arrow phloridzin was added. The dotted line shows the normal course of the fermentation. We have usually found that the course of fermentation after the addition of phloridzin is *linear*, in contrast to the effect of a substance such as allyl alcohol where the rate of evolution of gas after addition falls off quickly. (This may be, however, purely a matter of relative concentrations.)

### The influence of the mode of addition and the amount of substance.

In the experiments to be described the amount of substance under investigation added to the reaction vessel was either $10^{-3}$ or $10^{-4}$ of a g.-molecule dissolved or suspended in 4 cc. water. In one series of experiments glucosides ($10^{-3}$ g.-mol.) were added dissolved in 2 cc. ethyl alcohol. They all gave inhibitions, phloridzin retarding the initial rate of fermentation over 83 % whilst the others retarded to the extent of 40–50 %. A control experiment, however, showed that 2 cc. ethyl alcohol alone effected a retardation of 41 % (see Table IV). We deemed it advisable therefore not to add alcohol to the system, though it is clear from this experiment that phloridzin exerts a far greater effect than the other glucosides tested.

In considering the effect of alcohol and the linear rate of a normal zymin fermentation it should be remembered that to produce 1 cc. alcohol about 400 cc. $CO_2$ must be evolved. In our experimental time, a total of not more than 150 cc. $CO_2$ are evolved, so that sufficient alcohol has not been produced to make a serious alteration. Zeller [1927] has shown that small quantities of alcohol appear to accelerate rather than diminish the rate of fermentation of glucose by living yeast.

Table IV. *Influence of glucosides in alcoholic solution on zymin fermentation of glucose.* $10^{-3}$ *g.-mol. glucoside in* 2 *cc. ethyl alcohol.*

| Glucoside | Time in min. to produce 10 cc. $CO_2$ before addition | Time to produce 10 cc. $CO_2$ after addition | % retardation of initial rate | Final $p_H$ of reaction mixture |
|---|---|---|---|---|
| Phloridzin ... ... | 10·4 | 62 | 83·3 | 6·38 |
| Salicin ... ... | 10·5 | 19 | 44·8 | 6·1 |
| Amygdalin ... ... | 10·7 | 20·5 | 47·8 | 6·19 |
| Alcohol (control) 2 cc. | 11·7 | 20 | 41·5 | 6·0 |

Table V.

| Exp. A. Zymin F | | | Exp. B. Zymin F | | | Exp. C. Zymin X | | |
|---|---|---|---|---|---|---|---|---|
| $t$ | $CO_2$ | $CO_2/t$ | $t$ | $CO_2$ | $CO_2/t$ | $t$ | $CO_2$ | $CO_2/t$ |
| 9·4 | 10 | 1·06 | 10·6 | 10 | 0·94 | 9·1 | 10 | 1·1 |
| 22·9 | 20 | 0·87 | 22·1 | 20 | 0·90 | 18·9 | 20 | 1·06 |
| 28·6 | 25 | 0·87 | 27·76 | 25 | 0·90 | → 0·05 g. aesculin added | | |
| →$10^{-3}$ g.-mol. (0·446 g.) phloridzin added at this point to the reaction vessel | | | →$10^{-3}$ g.-mol. salicin added | | | 10·2 | 10 | 0·98 |
| | | | 13·2 | 10 | 0·76 | 20·3 | 20 | 0·98 |
| | | | 26·5 | 20 | 0·75 | → 0·04 g. phloridzin added | | |
| 25·3 | 10 | 0·39 | 33 | 25 | 0·76 | 17·0 | 10 | 0·59 |
| 52·9 | 20 | 0·38 | | | | 34·5 | 20 | 0·58 |
| 67·8 | 25 | 0·37 | | | | | | |

Table V gives a series of results carried out with two preparations of zymin (F and X). The effects of phloridzin and salicin are compared in the case of zymin F. Here the $CO_2/t$ quotient falls from 0·87 to 0·37 by the addition of $10^{-3}$ g.-mol. phloridzin, whilst the quotient drops from 0·90 to 0·76 for the addition of the same molar quantity of salicin. In the case of zymin X,

0·05 g. aesculin was added (in water) to the reaction vessel when the $CO_2/t$ quotient was 1·06, causing a fall of the quotient to 0·98. This addition was followed, in the same experiment, by the addition of 0·04 g. phloridzin whereon the quotient fell to 0·58.

### Experimental results with glucosides and polyhydric phenols.

Table VI gives a series of results demonstrating the effects of four glucosides, phenol and the polyhydric phenols on the zymin fermentation of glucose. The effects of $10^{-3}$ g.-mol. of these substances with zymin F and $10^{-4}$ g.-mol. with zymin M are shown. Phloretin[1] was also tested.

These results clearly indicate that the substances of outstanding inhibiting action are phloridzin, phloretin and phloroglucinol. In small quantities the effects of phloridzin and phloroglucinol relative to those of the other substances are even more marked, phloridzin having the greatest action. Its retardation of the initial rate of $CO_2$ production, under our experimental conditions, was 31 % for $10^{-4}$ g.-mol. and 59 % for $10^{-3}$ g.-mol.

The fermentation mixtures were examined at the termination of the experiments for change in $p_H$ (see Table VI). The final $p_H$ was very nearly the same (about 6·0) in most cases, and it is clear that the change in $p_H$ is not connected with the retardation of the rate of fermentation. In buffered solution phloridzin has the same retarding action.

Table VI. *Times (in minutes) taken after attainment of steady rates.*

| | Zymin F. $10^{-3}$ g.-mol. glucoside, etc. added | | | | Zymin M. $10^{-4}$ g.-mol. glucoside, etc. added | | |
|---|---|---|---|---|---|---|---|
| Glucoside, etc. | Time to produce 10 cc. $CO_2$ before addition | Time to produce 10 cc. $CO_2$ after addition | % retardation of initial rate | Final $p_H$ | Time to produce 10 cc. $CO_2$ before addition | Time to produce 10 cc. $CO_2$ after addition | % retardation of initial rate |
| Phloridzin | 11·5 | 28·0 | 59 | 6·0 | 4·5 | 6·5 | 31 |
| Salicin | 11·5 | 13·3 | 14 | 6·0 | 4·8 | 4·8 | 0 |
| Amygdalin | 11·6 | 13·8 | 16 | 5·8 | 4·5 | 4·5 | 0 |
| Aesculin | 11·0 | 15·5 | 29 | 6·0 | 4·5 | 4·6 | 2 |
| Phenol | 10·8 | 15·7 | 31 | 6·0 | 4·55 | 4·5 | 0 |
| Resorcinol | 11·5 | 16·2 | 29 | 6·1 | — | — | — |
| Quinol | 11·1 | 15·0 | 26 | 6·1 | 4·8 | 4·8 | 0 |
| Catechol | 11·2 | 15·5 | 28 | 6·0 | 4·8 | 4·7 | 0 |
| Pyrogallol | 10·8 | 14·2 | 24 | 6·7 | 4·9 | 4·9 | 0 |
| Phloroglucinol | 11·0 | 18·5 | 41 | 6·1 | 5·1 | 5·4 | 6 |
| Phloretin | 12·1 | 20·0 | 40 | — | — | — | — |

### Influence of phloridzin, etc. on the fermentation of pyruvic acid by zymin.

*Experimental method.* Conditions, here, differ from those of the glucose fermentation. It is necessary to use a buffer (acetate), as has been described by other workers [*e.g.* Neuberg, 1927], to secure good and reproducible results. The rate of fermentation is not linear. A typical curve is shown in Fig. 5.

[1] It is possible that the sample of phloretin we used contained a trace of phloridzin.

We find a good reaction mixture to consist of 8 g. zymin, 40 cc. water, 5 cc. 10 % pyruvic acid and 5 cc. 2*M* potassium acetate solution. The zymin is well shaken with the water and introduced into the reaction vessel where it is allowed to reach the temperature of the thermostat. The pyruvic acid and acetate buffer, also at the same temperature, are now added and readings commenced.

Since the rate of $CO_2$ production is not linear the most convenient method of ascertaining the effect of an added substance is to determine the time taken for the evolution of a given volume of $CO_2$ in the presence and in the absence of the substance in question.

The substance is added to the reaction vessel after the evolution of the first 10 cc. of $CO_2$ from the zymin-pyruvic acid mixture.

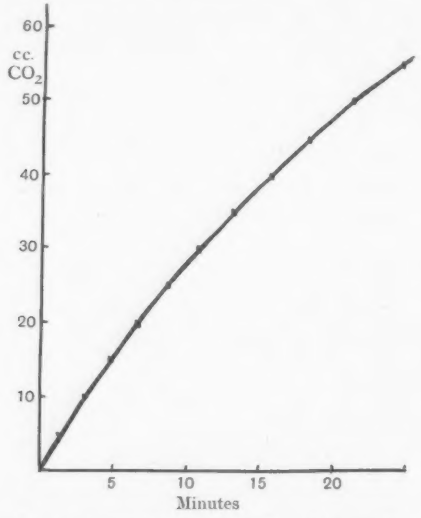


Fig. 6. Fermentation of pyruvic acid by zymin.

Table VII gives the results of three experiments which are typical. Exp. A gives the variation of the quotient $CO_2/t$ with time in a normal fermentation of pyruvic acid by zymin where 5 cc. water were added after the evolution of the first 10 cc. $CO_2$. Exp. B gives the values $CO_2/t$ where $10^{-3}$ g.-mol. phloridzin (suspended in 5 cc. water) was added after the production of the first 10 cc. gas. In Exp. C aesculin ($10^{-3}$ g.-mol.) was substituted for phloridzin. It will be seen that, within experimental error, phloridzin, like aesculin, has *no* perceptible retarding action on the $CO_2$ production from pyruvic acid. Table VIII shows that under these conditions (in the presence of acetate buffer) phloridzin has a marked effect on the fermentation of glucose by zymin, the $CO_2/t$ quotient falling from 2·1 to 0·86.

Table VII. *Fermentation of pyruvic acid by zymin.*

| Exp. A. 5 cc. water added to reaction mixture after first 10 cc. $CO_2$ evolved | | | Exp. B. $10^{-3}$ g.-mol. phloridzin added in 5 cc. water after first 10 cc. $CO_2$ evolved | | | Exp. C. $10^{-3}$ g.-mol. aesculin added in 5 cc. water after first 10 cc. $CO_2$ evolved | | |
|---|---|---|---|---|---|---|---|---|
| $t$ | $CO_2$ | $CO_2/t$ | $t$ | $CO_2$ | $CO_2/t$ | $t$ | $CO_2$ | $CO_2/t$ |
| 3·25 | 10 | 3·1 | 3·9 | 10 | 2·6 | 4·1 | 10 | 2·4 |
| 9·1 | 20 | 2·2 | 8·8 | 20 | 2·3 | 8·9 | 20 | 2·2 |
| 14·75 | 30 | 2·0 | 14·4 | 30 | 2·1 | 14·7 | 30 | 2·0 |
| 18·0 | 35 | 1·9 | 20·9 | 40 | 1·9 | — | — | — |

Table VIII. *Fermentation of glucose by zymin in presence of acetate buffer. Times taken after attainment of steady rate.*

| $t$ | $CO_2$ | $CO_2/t$ |
|---|---|---|
| 4·8 | 10 | 2·1 |
| 9·65 | 20 | 2·1 |
| 14·3 | 30 | 2·1 |
| → $10^{-3}$ g.-mol. phloridzin added in 5 cc. water | | |
| 9·16 | 10 | 1·1 |
| 19·7 | 20 | 1·1 |
| 31·2 | 30 | 0·96 |
| 46·4 | 40 | 0·86 |

None of the glucosides or phenols which we have tested has any inhibitory action, within experimental error, on the pyruvic acid fermentation by zymin. Table IX summarises these results.

Table IX. *Fermentation of pyruvic acid by zymin.*

| Glucoside, etc. ($10^{-3}$ g.-mol.) added in 5 cc. water to reaction mixture, after evolution of first 10 cc. $CO_2$ | Time in min. to produce 30 cc. $CO_2$ |
|---|---|
| Phloridzin | 14·4 |
| Amygdalin | 13·0 |
| Salicin | 13·5 |
| Aesculin | 14·7 |
| Phenol | 14·2 |
| Resorcinol | 15·9 |
| Catechol | 15·7 |
| Phloroglucinol | 14·7 |
| Pyrogallol | 14·7 |
| Water (control) alone | 14·7 |

### Experiments with live yeast.

150 g. yeast were crumbled and suspended in one litre of water. The suspension was centrifuged, washed three times and finally shaken with 350 cc. water. A good reaction mixture for glucose fermentation consisted of 6 cc. of the yeast suspension, 4 g. glucose and 34 cc. water. A typical curve for the fermentation is shown in the first part of Fig. 7.

Addition of phloridzin has no perceptible effect on the fermentation of glucose by live yeast. This is indicated in Table X, where Exp. A shows the effect of addition of 6 cc. water, after the attainment of a steady rate, and Exp. B the effect of adding $10^{-3}$ g.-mol. phloridzin suspended in 6 cc. water.

It will be observed that the addition of phloridzin causes no change in the $CO_2/t$ quotient. Table XI summarises results obtained with various additions in the fermentation of glucose by live yeast.

Phloridzin has no perceptible action on the fermentation of pyruvic acid by live yeast.

Table X. *Fermentation of glucose by live yeast. Action of phloridzin.*

| Exp. A | | | Exp. B | | |
|---|---|---|---|---|---|
| $t$ | $CO_2$ | $CO_2/t$ | $t$ | $CO_2$ | $CO_2/t$ |
| 6·8 | 10 | 1·5 | 7·15 | 10 | 1·4 |
| 13·4 | 20 | 1·5 | 13·7 | 20 | 1·5 |
| 20·5 | 30 | 1·5 | 20·2 | 30 | 1·5 |
| 6 cc. water added to the reaction mixture at this point | | | $10^{-3}$ g.-mol. phloridzin in 6 cc. of water added | | |
| 6·3 | 10 | 1·6 | 7·4 | 10 | 1·4 |
| 12·9 | 20 | 1·6 | 14·1 | 20 | 1·4 |
| 19·2 | 30 | 1·6 | 20·7 | 30 | 1·4 |
| 25·7 | 40 | 1·6 | 27·4 | 40 | 1·5 |

Table XI. *Fermentation of glucose by live yeast.*

| Glucoside, etc. added $10^{-4}$ g.-mol. | Time to produce 40 cc. $CO_2$ before addition | Time to produce 40 cc. $CO_2$ after addition |
|---|---|---|
| Phloridzin | 14·0 | 14·1 |
| Aesculin | 13·8 | 13·8 |
| Salicin | 13·8 | 13·6 |
| Amygdalin | 13·6 | 13·7 |
| Phloretin | 13·6 | 13·6 |
| Phloroglucinol | 13·5 | 13·8 |

## The effects of allyl alcohol, etc. on the fermentation of glucose.

Allyl alcohol has a retarding action which increases with time on both zymin and live yeast fermentations of glucose (Fig. 7). Acrylic acid has a similar action but we have observed no perceptible retarding effects with allylamine or allylacetic acid.

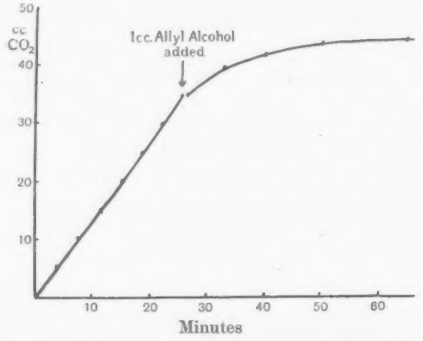


Fig. 7. Effect of allyl alcohol on fermentation of glucose by live yeast.

SUMMARY.

1. Phloridzin shows a much higher retarding action on the normal rate of zymin fermentation of glucose than salicin, aesculin or amygdalin, this being especially marked when relatively small quantities of the substances are used. Under our experimental conditions $10^{-4}$ g.-mol. phloridzin effects a 31 % retardation and $10^{-3}$ g.-mol. a 59 % retardation of the rate of fermentation due to zymin. Phloretin shows a similar but somewhat less vigorous action than phloridzin. The rate of fermentation after addition of phloridzin is linear.

2. Of the polyhydric phenols, phloroglucinol (which is the phenol present in phloridzin) is the most effective in retarding the rate of fermentation of glucose by zymin.

3. The retarding action of phloridzin is not exhibited in the zymin fermentation of pyruvic acid, or in the fermentations of glucose and pyruvic acid by live yeast.

4. Allyl alcohol and acrylic acid retard the fermentation of glucose by zymin and live yeast, the retardation increasing with time. Neither allylamine nor allylacetic acid has such an action under the conditions of our experiments.

We are grateful to Sir F. G. Hopkins for the interest he has taken in this work, and to Dr G. Jessop for his kindness in lending us his apparatus.

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# XXXV. THE VALUE OF WHOLE POTATO IN HUMAN NUTRITION[1].

By STANISŁAW KAZIMIERZ KON and ANIELA KLEIN.

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(*Received December 29th, 1927.*)

The potato is a very important constituent of the diet of many civilised nations, and it may be considered to furnish, with bread, the bulk of the food of the rural population of such countries as Poland and Russia. There is little doubt that the Polish or Russian peasant is nevertheless very healthy and able to do extremely hard work under trying conditions. Deficiency diseases such as beriberi, pellagra or scurvy are practically unknown in Poland.

For anybody acquainted with the diet and mode of life of the Polish peasant the high value of the potato as food would seem *a priori* to be well established. Students of nutritional problems, however, have very discrepant opinions concerning the value of potato. A careful review of earlier works and views relating to this problem is to be found in the papers of Hindhede [1913, 1, 2]. These are also the only experiments, so far as we are aware, which extend over long periods of time. It is well known that Hindhede and his collaborators were able to live over long periods of time on a diet in which the nitrogen was solely derived from whole potato. The experiments carried out both by his opponents and those who confirmed his opinions were of very short duration [Abderhalden, *et al.*, 1915; Lauter and Jenke, 1925; Rose and Cooper, 1917]. We therefore thought it advisable to repeat the investigations of Hindhede, continuing the experiment for several months. It was not primarily our intention to verify the contention of Hindhede concerning the question of potato minimum, but to see whether the potato would prove sufficient for the human adult as practically the sole source of nitrogen and the main source of calories.

The subjects were two healthy adults, a man aged 25, and a woman aged 28. They are subsequently referred to as (A) and (B) respectively. During the experiment they both did laboratory work, and (A) was also engaged in various sports. The diet consisted of potatoes, supplemented with butter or pork fat with the addition of a few fruits (apples and pears); tea or black coffee and sugar were also occasionally taken. The amount of fat consumed was not accurately estimated, it varied from 120–150 g. daily.

[1] This work was carried out in 1925, but the publication has been unavoidably delayed.

The potatoes were either steam-cooked unpeeled and then consumed with butter and salt, or fried. They were sometimes mashed after cooking, or sliced and made into salad with a little oil. In the later stages of the experiment it was found, however, that simple steam-cooked potatoes seemed the most palatable and this method of preparation was therefore generally used. Three meals daily were taken. The potatoes were weighed out for each meal and an endeavour was made to keep the food intake constant throughout the experiment. From time to time nitrogen balance periods were instituted. Urine was then collected and analysed daily, faeces were analysed in 2 or 3 day samples. Each metabolism period lasted 4 days, except period 4 of subject (A), which lasted 6 days. The whole experiment extended over 167 days, from Dec. 4, 1924 till May 20, 1925. The results of the nitrogen balance estimations are given in Tables I and II. The nitrogen content of the potatoes was 0·34 %.

Table I. *Subject (A).*

| | Date | Daily intake of potatoes g. | Food-N g. | Urinary N g. | Faecal N g. | Total N excreted g. | N balance |
|---|---|---|---|---|---|---|---|
| Period I | 22. xii. 24 | 1575 | 5·35 | 2·98 | 1·31 | 4·29 | |
| | 23. xii. 24 | 1500 | 5·10 | 3·63 | 1·31 | 4·94 | |
| | 24. xii. 24 | 1557 | 5·29 | 3·86 | 1·92 | 5·78 | |
| | 25. xii. 24 | 1610 | 5·47 | 3·79 | 1·92 | 5·71 | |
| Daily average | | 1560 | 5·30 | 3·56 | 1·61 | 5·18 | +0·12 |
| Period II | 12. i. 25 | 1770 | 6·02 | 3·07 | 2·32 | 5·39 | |
| | 13. i. 25 | 1746 | 5·94 | 2·77 | 2·32 | 5·09 | |
| | 14. i. 25 | 1758 | 5·98 | 3·11 | 2·05 | 5·16 | |
| | 15. i. 25 | 1758 | 5·98 | 3·56 | 2·05 | 5·61 | |
| Daily average | | 1758 | 5·98 | 3·13 | 2·18 | 5·31 | +0·67 |
| Period III | 2. ii. 25 | 1624 | 5·52 | 3·06 | 1·47 | 4·53 | |
| | 3. ii. 25 | 1638 | 5·57 | 3·70 | 1·47 | 5·17 | |
| | 4. ii. 25 | 1720 | 5·85 | 3·53 | 2·36 | 5·89 | |
| | 5. ii. 25 | 1661 | 5·65 | 3·52 | 2·36 | 5·88 | |
| Daily average | | 1661 | 5·65 | 3·45 | 1·91 | 5·37 | +0·28 |
| Period IV | 15. v. 25 | 1741 | 5·92 | 2·55 | 2·01 | 4·56 | |
| | 16. v. 25 | 1725 | 5·86 | 2·56 | 2·01 | 4·57 | |
| | 17. v. 25 | 1794 | 6·10 | 2·11 | 2·01 | 4·12 | |
| | 18. v. 25 | 1736 | 5·90 | 2·92 | 2·12 | 5·04 | |
| | 19. v. 25 | 1764 | 6·00 | 2·58 | 2·12 | 4·70 | |
| | 20. v. 25 | 1752 | 5·96 | 3·18 | 2·12 | 5·30 | |
| Daily average | | 1752 | 5·96 | 2·65 | 2·06 | 4·71 | +1·25 |

It will be seen that nitrogen equilibrium was attained by both subjects, by (A) more rapidly than by (B).

Body weight was nearly constant during the whole experiment. The sudden loss of weight of subject (A) towards the end of the experiment was perhaps associated with the beginning of a more intensive training (Table III).

The digestion was excellent throughout the experiment and both subjects felt very well. They did not tire of the uniform potato diet and there was no craving for change.

Table II. *Subject* (*B*).

| | Date | Daily intake of potatoes g. | Food-N g. | Urinary N g. | Faecal N g. | Total N excreted g. | N balance |
|---|---|---|---|---|---|---|---|
| Period I | 22. xii. 24 | 1075 | 3·65 | 3·62 | 1·60 | 5·22 | |
| | 23. xii. 24 | 1005 | 3·42 | 3·74 | 1·60 | 5·34 | |
| | 24. xii. 24 | 1115 | 3·79 | 3·64 | 2·03 | 5·67 | |
| | 25. xii. 24 | 900 | 3·06 | 3·22 | 2·03 | 5·25 | |
| Daily average | | 1024 | 3·48 | 3·55 | 1·81 | 5·37 | −1·89 |
| Period II | 12. i. 25 | 1330 | 4·52 | 3·54 | 1·70 | 5·24 | |
| | 13. i. 25 | 1230 | 4·18 | 2·21 | 1·70 | 3·91 | |
| | 14. i. 25 | 1293 | 4·39 | 2·92 | 1·16 | 4·08 | |
| | 15. i. 25 | 1318 | 4·48 | 3·73 | 1·16 | 4·88 | |
| Daily average | | 1293 | 4·39 | 3·10 | 1·43 | 4·53 | −0·14 |
| Period III | 2. ii. 25 | 1112 | 3·78 | 4·03 | 1·57 | 5·60 | |
| | 3. ii. 25 | 1200 | 4·08 | 3·37 | 1·57 | 4·94 | |
| | 4. ii. 25 | 1305 | 4·44 | 3·02 | 1·67 | 4·69 | |
| | 5. ii. 25 | 1050 | 3·57 | 2·64 | 1·67 | 4·31 | |
| Daily average | | 1167 | 3·97 | 3·26 | 1·62 | 4·88 | −0·91 |
| Period IV | 15. v. 25 | 925 | 3·14 | 2·27 | 0·90 | 3·17 | |
| | 16. v. 25 | 1008 | 3·43 | 2·40 | 0·90 | 3·30 | |
| | 17. v. 25 | 979 | 3·33 | 2·70 | 0·87 | 3·57 | |
| | 18. v. 25 | 1243 | 4·22 | 2·84 | 0·87 | 3·71 | |
| Daily average | | 1039 | 3·53 | 2·55 | 0·88 | 3·44 | +0·09 |

Table III.

| Date ... ... | | Dec. 4 | Dec. 18 | Jan. 2 | Jan. 16 | Jan. 30 | Feb. 13 | Feb. 27 |
|---|---|---|---|---|---|---|---|---|
| Body-weight kg. | A | 64·6 | 64·0 | 62·5 | 62·6 | 62·9 | 64·1 | 64·6 |
| | B | 63·5 | 62·8 | 62·5 | 62·3 | 62·4 | 61·8 | 61·3 |
| Date ... ... | | Mar. 13 | Mar. 22 | Apr. 11 | Apr. 24 | May 9 | May 16 | |
| Body-weight kg. | A | 64·3 | 64·5 | 64·9 | 64·2 | 62·7 | 61·3 | |
| | B | 60·8 | 61·3 | 61·8 | 61·8 | — | 61·7 | |

We think that our results must be looked upon as a confirmation of the experiment of Hindhede, and the high value of potato as a source of nitrogen for the human adult seems to be confirmed.


## Summary.

An experiment is described in which two adults, a man and a woman, lived over a period of 167 days in nitrogen equilibrium and in good health on a diet in which the nitrogen was practically solely derived from the potato. The daily nitrogen intake was on the average 5·7 g. for the man and 3·8 g. for the woman.


This work was done under the direction of Dr Casimir Funk to whom our thanks are due.

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# XXXVI. THE NUTRITIONAL VALUE OF TUBERIN, THE GLOBULIN OF POTATO.

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(*Received December 29th, 1927.*)

There is much controversy concerning the value of potato as a source of nitrogen, and especially divergent opinions are encountered when the results of human maintenance experiments are compared with experiments carried out on growing rats. The high value of potato nitrogen for human beings seems to be established beyond doubt, not only by the experiments of Hindhede [1913, 1, 2; 1917], Rose and Cooper [1917], and Kon and Klein [1928], but also by the large scale natural experiment of potato-eating nations. On the other hand, McCollum, Simmonds and Parsons [1918] and Hartwell [1927] state that the potato is not a good source of nitrogen for the rat, young rats either growing very slowly or failing to grow on a diet in which the nitrogen was solely derived from whole potato. These rats also had bad fur. Adult rats, however, could be maintained in fair condition for a period of 9 months on a diet consisting of potato supplemented with NaCl, $CaCO_3$ and butter fat. In the opinion of the former investigators "maintenance on 8 % of potato protein (N × 6·27) compares favourably with that obtained with a diet properly adjusted but containing only 7 % of wheat or oat protein."

All these experiments relate to the total nitrogen of potato. According to König [1903] only 43–65 % of the potato nitrogen is of protein nature. In view of these facts it seemed worth while to investigate the nutritional value of the isolated potato protein.

The chief, and probably only, protein of potato is a globulin called tuberin by Osborne and Campbell [1896].

## Experimental.

### *Preparation of the protein.*

The method described by Sjöllema and Rinkes [1912] was first used. The pressed and filtered potato-juice was saturated with common salt, the precipitate purified by solution in 10 % NaCl and dialysis, and then dried in the usual way. This method was associated with heavy losses, due chiefly to incomplete precipitation of the protein by the salt, incomplete solubility of the precipitate in 10 % NaCl, and extremely slow and incomplete precipitation during dialysis. Some 200 g. of protein were obtained in this way.

This batch was very dark in colour. Feeding experiments with this resulted in complete failure. When the protein was incorporated in an otherwise complete diet and fed to young rats at a 10 % level there was no growth at all, a marked loss of appetite was soon manifested and the rats died in the course of a few days, rarely surviving one week. They were always in a pitiful condition, thin and emaciated and continuously losing weight. When caseinogen replaced the potato protein in the diet the rats grew normally. On post-mortem examination of the potato-fed rats retention of urine was frequently found, but nothing else of interest. The symptoms clearly pointed to the presence of some toxic substance in the protein and it was thought that this might be solanin. There is much controversy regarding the toxicity of solanin and its rôle in cases of so-called potato poisoning. In any case, when taken in concentrations found in good sound potatoes it is obviously absolutely harmless to man and animals. It is therefore probable that if solanin is the cause of the symptoms of poisoning it must be concentrated along with the tuberin in the course of preparation of the latter. By extracting a 70 g. portion of the tuberin three times with boiling 85 % alcohol and treating the extract as described later in this paper, 0·1 g. of solanin, identified by the various colour reactions, was obtained. The potatoes used were old, and were sprouting when extracted (May, 1927). In view of these facts it was decided to prepare the protein by heat coagulation, and the following method was finally adopted: the potatoes (new "Eclipse" potatoes) after careful washing were finely minced in a meat mincer in 12·5 kg. batches and pressed in a hand press through cheese cloth, the juice was collected under toluene, the residue reminced and pressed in a hydraulic press. The combined pressings were then filtered clear through a thick mat of filter pulp on a Büchner funnel, the mat being changed 2–3 times. The yield was between 9–10 l. from 12·5 kg. of potatoes. The clear filtrate was then placed in an enamelled iron pan and steam introduced until the temperature reached 90°, when the fluid was allowed to settle. The bulk of the clear fluid was then syphoned off and the residue transferred to a Büchner funnel and sucked dry. It was then shaken with distilled water, filtered, suspended in 85 % alcohol and boiled under a reflux condenser for 20 minutes. This was repeated twice. The residue was then dried in the usual way by suspending in increasing concentrations of alcohol and finally in absolute alcohol and ether. It was found most convenient to make the suspension by shaking the protein with the fluids on a mechanical shaker. After washing with absolute ether the protein was kept in a vacuum desiccator until sufficiently dry, then powdered and dried in a steam-oven for 3 hours at 95°. 192 kg. of potatoes yielded 553 g. of a very light grey powder containing 14·8 % N, 1·1 % S, and 1·8 % ash. The 85 % alcoholic extracts were evaporated *in vacuo* to a small bulk, acidified with sulphuric acid, heated on a water-bath and filtered. The filtrate was then made alkaline with ammonia and a gelatinous precipitate was formed after a few minutes' heating. This was filtered off, extracted with

hot 85 % alcohol, the alcohol evaporated on the water-bath, the residue redissolved in acidified water and precipitated by adding an excess of ammonia. The white gelatinous flakes were filtered off and dried. They had a very bitter taste and gave all the colour reactions of solanin. The yield from 533 g. of crude tuberin was 0·3 g.

### Feeding experiments.

Of the various methods employed for estimating or comparing the nutritive values of proteins, two seem to be more generally adopted. One of them, originated by Thomas [1909] and based on the estimation of nitrogen balance, is known in its present greatly improved form as the method of Mitchell [Mitchell, 1924, 1; Mitchell and Carman, 1926]. The other, relating the nitrogen intake to growth, has been evolved by Osborne, Mendel and Ferry [1919]. Both use rats as experimental animals. It was thought advisable to make use of both of them in the investigation of the nutritional value of tuberin, not only in order to obtain more reliable results, but also to gain a direct comparison of both biological methods.

#### *Mitchell's method.*

Six young pure-bred piebald rats were used. Their initial weights were 50–70 g. The methods of Mitchell were followed with a few small modifications. The cages were higher than those described by Mitchell and the screen supporting the rat was raised 5 cm. above the bottom, thus preventing any possibility of ingestion of filter-paper. The food was placed in special feeders and scattering almost entirely avoided. The faeces were collected daily, urine every second day. For cleaning the rats were transferred to fresh cages and any loss of urine or faeces thus avoided. The urine was washed out with 400 cc. of very hot weak sulphuric acid. The faeces were collected under acidified alcohol. The estimations were carried out on the weekly samples. The composition of the diets is given in Table I.

Table I.

| | Egg diet g. | Tuberin diet g. | Caseinogen diet g. |
|---|---|---|---|
| Tuberin | — | 8·5 | — |
| Caseinogen | — | — | 8·6 |
| Dried egg | 5·35 | — | — |
| Butter fat | 17 | 17 | 17 |
| Rice starch | 45·65 | 42·5 | 42·4 |
| Potato starch | 10 | 10 | 10 |
| Cane sugar | 17 | 17 | 17 |
| Salts | 5 | 5 | 5 |

Each rat received, separately from the diet, 2 drops of cod-liver oil and an aqueous extract of yeast equivalent to 0·3 g. daily. The daily intake of 3·7 mg. N from this source was not taken into consideration when calculating the nitrogen balance. The rations were prepared in quantities of 1 kg. and kept in tightly stoppered glass jars in a cool place. The potato starch added to the diet took the place of roughage owing to its low digestibility.

Table II. *Nitrogen metabolism data.*

*First group of rats.*

| Rat No. | Initial weight g. | Final weight g. | Daily food intake g. | Daily N intake mg. | Faecal N mg. | Body-N in faeces mg. | Food-N in faeces mg. | Absorbed N mg. | N in urine mg. | Body-N in urine mg. | Food-N in urine mg. | Food-N retained mg. | Biological value |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Low egg-nitrogen ration (0·71 % N) | | | | | | | |
| 1 | 70 | 76 | 8·00 | — | 22 | 2·8* | — | — | 18 | 25† | — | — | — |
| 2 | 63 | 69 | 8·05 | — | 22 | 2·7* | — | — | 14 | 22† | — | — | — |
| 3 | 62·5 | 63·5 | 6·53 | — | 18 | 2·8* | — | — | 14 | 22† | — | — | — |
| | | | | | | Tuberin ration (1·29 % N) | | | | | | | |
| 1 | 82 | 90 | 8·11 | 104 | 23 | 22 | 1 | 103 | 45 | 20 | 25 | 78 | 76 |
| 2 | 77 | 81 | 6·82 | 88 | 24 | 18 | 6 | 82 | 30 | 17 | 13 | 69 | 84 |
| 3 | 71 | 80 | 7·21 | 93 | 22 | 19 | 3 | 90 | 34 | 16 | 18 | 72 | 80 |
| | | | | | | Caseinogen ration (1·29 % N) | | | | | | | |
| 1 | 94 | 100 | 8·08 | 104 | 26 | 21 | 5 | 99 | 59 | 22 | 37 | 62 | 63 |
| 2 | 84 | 89 | 7·04 | 91 | 20 | 19 | 1 | 90 | 54 | 17 | 37 | 53 | 59 |
| 3 | 86 | 92 | 8·15 | 105 | 22 | 20 | 2 | 103 | 60 | 17 | 43 | 60 | 58 |
| | | | | | | Low egg-nitrogen ration (0·71 % N) | | | | | | | |
| 1 | 103 | 111 | 8·71 | — | 21 | 2·5* | — | — | 21 | 20† | — | — | — |
| 2 | 92 | 97 | 7·57 | — | 20 | 2·7* | — | — | 18 | 19† | — | — | — |
| 3 | 97 | 104 | 8·79 | — | 22 | 2·5* | — | — | 18 | 18† | — | — | — |

*Second group of rats.*

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Low egg-nitrogen ration (0·71 % N) | | | | | | | |
| 4 | 47 | 53 | 6·48 | — | 19 | 2·9* | — | — | 13 | 26† | — | — | — |
| 5 | 55·5 | 58·5 | 6·15 | — | 19 | 3·1* | — | — | 14 | 25† | — | — | — |
| 6 | 61·5 | 65·5 | 7·00 | — | 20 | 2·9* | — | — | 14 | 23† | — | — | — |
| | | | | | | Caseinogen ration (1·29 % N) | | | | | | | |
| 4 | 62 | 69·5 | 6·70 | 86 | 23 | 20 | 3 | 83 | 29 | 15 | 14 | 69 | 83 |
| 5 | 64 | 70 | 6·57 | 85 | 22 | 20 | 2 | 83 | 37 | 16 | 21 | 62 | 75 |
| 6 | 72 | 76 | 6·97 | 90 | 22 | 19 | 3 | 87 | 42 | 16 | 26 | 61 | 70 |
| | | | | | | Tuberin ration (1·29 % N) | | | | | | | |
| 4 | 75 | 78 | 6·31 | 81 | 20 | 18 | 2 | 79 | 50 | 16 | 34 | 45 | 57 |
| 5 | 75 | 78 | 6·55 | 84 | 29 | 19 | 10 | 74 | 46 | 17 | 29 | 45 | 61 |
| 6 | 84 | 90 | 7·54 | 97 | 26 | 20 | 6 | 91 | 48 | 18 | 30 | 61 | 67 |
| | | | | | | Low egg-nitrogen ration (0·71 % N) | | | | | | | |
| 4 | 81 | 84 | 7·47 | — | 22 | 2·9* | — | — | 15 | 18† | — | — | — |
| 5 | 82 | 88 | 7·87 | — | 22 | 2·8* | — | — | 17 | 20† | — | — | — |
| 6 | 91·5 | 97 | 8·43 | — | 22 | 2·6* | — | — | 18 | 19† | — | — | — |

* These figures represent the metabolic faecal nitrogen per g. of food consumed and are used in estimating the body-N in the faeces in the intermediate periods from the amounts of food consumed.

† These figures represent the endogenous urinary N per 100 g. of body weight and are used in estimating the body-N in the urine in the intermediate periods from the average body weight.

Table III. *Summary of biological values.*

| Rat No. ... | 1 | 2 | 3 | 4 | 5 | 6 | Average |
|---|---|---|---|---|---|---|---|
| Tuberin diet | 76 | 84 | 80 | 57 | 61 | 67 | 71 |
| Caseinogen diet | 63 | 59 | 58 | 83 | 75 | 70 | 68 |

*Method of Osborne, Mendel and Ferry.*

The protein was fed at three levels of intake, namely 8·5, 10·5 and 12·7 %. Rice starch was added to 51 and the composition of the rest of the diet was as stated in Table I. According to Osborne and Campbell [1896] tuberin contains 16·24 % N so that the actual amounts present in the above diets were 7·8, 9·7, 11·8 g. respectively (N × 6·16).

Young pure-bred piebald rats were used. They were kept in individual screened cages. The food was mixed with a little water and placed in tall, narrow porcelain jars to avoid scattering. It was offered slightly in excess of the intake. Each rat received separately from the diet 2 drops of cod-liver oil and aqueous extract of yeast equivalent to 0·4 g. daily.

At the end of the experiment all the rats were in very good condition and their fur was smooth and soft. It is possible that the maximal gain per g. of protein ingested would be found still below the 7·8 % level and it is to be regretted that the protein was not fed at a lower level.

Table IV.

| Diet | Rat | Sex | Days | Total intake: Food g. | Total intake: Protein g. | Gain in weight g. | Gain per g. protein g. |
|---|---|---|---|---|---|---|---|
| 8 % tuberin | 7 | F. | 30 | 260 | 20·4 | 40·0 | 2·0 |
| | 8 | M. | 30 | 218 | 17·1 | 33·5 | 2·0 |
| 10 % tuberin | 9 | F. | 30 | 233 | 22·6 | 43·5 | 1·9 |
| | 10 | F. | 30 | 235 | 22·8 | 32·5 | 1·4 |
| | 11 | M. | 28 | 203 | 19·6 | 32·0 | 1·6 |
| | 12 | M. | 30 | 213 | 20·6 | 34·5 | 1·7 |
| | 13 | M. | 28 | 201 | 19·5 | 33·0 | 1·7 |
| 12 % tuberin | 14 | F. | 16* | 121 | 14·3 | 25·0 | 1·7 |
| | 15 | F. | 28 | 212 | 24·9 | 42·5 | 1·7 |

* This rat was taken ill on the 17th day.

## Discussion.

It would appear from the above data that tuberin is a good and complete protein. The fact that it is sufficient not only for maintenance, but also for continued though subnormal growth when fed at the low level of 7·8 %, places it among the best of the isolated vegetable proteins. The gain per g. of protein is at the 7·8 % level distinctly higher than that found by Osborne, Mendel and Ferry [1919] for caseinogen at a 9·3 % level. Moreover, the nutritional value of isolated tuberin compares very favourably with values found by Osborne and Mendel [1920] for the entire proteins of barley, oat, rye and wheat kernels at an 8–10 % level. The biological value of the entire nitrogen of the potato has been estimated by Mitchell [1924, 2] at two levels of intake, called approximately 5 and 10 % protein (N × 6·25). In reality these levels were found after calculation to be 5·5 and 7·9 % respectively. It is therefore possible to compare Mitchell's higher level with the results found in the present work for the isolated protein. The difference between the biological values (67 for the entire potato nitrogen and 71 for tuberin) is well within the limits of experimental error. It would therefore appear, as judged by Mitchell's methods, that the non-protein nitrogenous constituents of the potato are well utilised by the rat for repair of the body tissues. The value of potato-nitrogen for growth of young rats has been estimated by McCollum, Simmonds and Parsons [1918] and Hartwell [1927]. The former workers found that when whole cooked dried potato was supplemented with

butter fat and Na, Ca and Cl, and fed at a protein (N × 6·25) level of approximately 8 %, some growth was obtained in a few rats over a period of 16 weeks, but the rats were emaciated and in poor condition. Other rats failed to grow on this diet. The authors ascribe their results to the deficiency and poor quality of the protein of the diet. It might be argued that the diet was not optimally balanced with regard to the other constituents and especially it has not been demonstrated that NaCl and $CaCO_3$ are alone sufficient to make good the inorganic deficiencies of potato. Hartwell by feeding cooked potato (containing probably approximately 75 % water) supplemented by the addition of 5 % butter and 1 % salt mixture, obtained better growth than McCollum and his co-workers. It was nevertheless very subnormal and the animals had at a certain period very bad fur. Hartwell thinks that the protein deficiency of potato when fed to rats is rather of a quantitative than a qualitative nature. The amount of fat present (some 20 % of the dry substance) might tend to lower the food intake by increasing the caloric value. That this is really the case seemed to be proved by the present writer by feeding to young rats at first a diet similar to that employed by Hartwell (100 g. of steamed potatoes, 5 g. of butter, 1 g. of salt mixture) with the addition of 2 drops of cod-liver oil and 0·5 cc. of an alcoholic extract of marmite. The rats grew very slowly, and reached a plateau after having gained about 30 g. The butter was then reduced to 1 %; there was then a rapid response, growth was resumed and the fur became better. After a month the general condition of the rats was very markedly improved. It would seem that when the other constituents of the ration are properly adjusted and the caloric value is low, the nitrogen of potato enables young rats to grow at a subnormal rate over long periods of time. It is to be regretted that the food intake of rats receiving whole potatoes has not been determined, as it was therefore impossible to calculate the nutritional value of whole potato-nitrogen according to the method of Osborne, Mendel and Ferry. Judging by the rate of growth of the rats, their behaviour and general appearance, there is little doubt however that the isolated protein is at the same level of intake distinctly superior to the entire nitrogen of potato. It is interesting to note that the difference disappears when the method of Mitchell is used and it seems evident that both methods often give quite discordant results. Thus, for example, the biological values at an 8 to 10 % level of veal, beef, pork, oat, wheat, and potato proteins were found by Mitchell [Mitchell and Carman, 1924, 1926; Mitchell, 1924, 2] to be equal to 62, 69, 74, 65, 67 and 67 respectively, with no significant differences between the various proteins, whereas using the method of Osborne, Mendel and Ferry the following gains per g. of protein ingested have been found: veal 2·9 g. [Hoagland and Snider, 1926, 1]; beef 3·1, pork 2·8 g. [Hoagland and Snider, 1926, 2]; oats 1·3 g., wheat 1·3 g. [Osborne and Mendel, 1920]; tuberin 1·8 g. (present work). Here the difference is very marked and decidedly in favour of the animal proteins. A specific error seems to be inherent in the method based

on the estimation of nitrogen balance, namely, the tendency to give higher biological values in the periods immediately following the standardising nitrogen-free or low nitrogen periods. This is specially marked in the present work, but may also be found in the papers of Mitchell and his co-workers [especially Mitchell and Beadles, 1927] and of Klein, Harrow, Pine and Funk [1926]. The method of Osborne, Mendel and Ferry also has the advantage of greater simplicity. On the other hand, it requires roughly three times as much of the protein for the same number of rats as the method of Mitchell, and is more sensitive to variations of the food intake. Obviously the two methods are not strictly comparable, as the one measures the efficiency of a given protein to make good, over short periods of time, the wear and tear of body tissues, while the other measures the ability of a protein to build new tissues. The question which of these methods is the more adequate for judging the relative values of various proteins in human nutrition by means of animal feeding experiments remains to be answered.

SUMMARY.

The biological value of tuberin, the globulin of potato, was found to be equal to 71 as measured at an 8 % level of intake by the method of Mitchell. The protein was also fed to rats at levels of approximately 8, 10 and 12 %, and a maximal gain of 2 g. body weight per g. of protein ingested was found at a 7·8 % level of intake by the method of Osborne, Mendel and Ferry. The protein was not fed at lower levels. Tuberin appears to be a good, complete protein.

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# XXXVII. ON THE EVIDENCE FOR PHOSPHATIDES IN THE EXTERNAL SURFACE OF THE PLANT PROTOPLAST.

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## Introduction.

During recent years Hansteen Cranner [1910, 1914, 1919, 1922, 1925, 1926], and subsequently Grafe and his co-workers [1925, 1926, 1927], have published results of the examination of the substances leached into aqueous liquids from living parenchymatous plant tissues. On the assumption that such substances must come from the external surface of the protoplast, or from the cell wall, as the semipermeability of the protoplast is still maintained, these experiments have been widely quoted as evidence for the presence of phosphatides in the external protoplasmic layer or "plasma membrane." This conclusion does not necessarily follow and will be critically examined elsewhere. The present paper is confined to a brief record of a somewhat extensive series of such leaching experiments in which it has proved impossible to substantiate the presence of appreciable quantities of phosphatides in the aqueous extracts.

## Leaching experiments on storage tissues.

The substances obtained by leaching cut slices of Scotch seed potatoes, variety Sharpe's Express, were first examined. The detailed description of the procedure and examination of products will be omitted as it is substantially the same as that to be described later. As the result of the examination of the accumulated basic lead acetate precipitate (100 g.), and also the filtrates evaporated and freed from lead, derived from leaching 6–8 kg. of fresh, washed slices of tissue, a turbid aqueous extract was obtained which contained calcium, magnesium and potassium, and the anions chloride, phosphate, and carbonate, and only reduced Fehling's solution slightly, even after inversion with mineral acid. On warming, some coagulation occurred, and the coagulum contained both nitrogen and phosphorus, and gave biuret and xanthoproteic colour reactions. The turbid solution, enclosed in a parchment dialyser and dialysed against distilled water, yielded inside the dialyser a gelatinous solid which also contained nitrogen and phosphorus and was probably a globulin.

The total amount of crude ether-soluble fatty acid which could be obtained by sulphuric acid hydrolysis of 100 g. of the lead acetate precipitate was 0·5 g. By repeated crystallisation this yielded a small amount of a white, solid, fatty acid of low melting point, which was, however, inadequate for further investigation. No ether-soluble unsaponifiable material was obtained, but a small amount of ether-insoluble acid similar to that isolated by Rhodes [1925] from potato cork was noticed. In spite of a careful examination for nitrogen bases (other than the more complex amino-acids which might be obtained from protein hydrolysis) amongst the hydrolytic products of the lead acetate precipitate, no evidence could be obtained even of traces of choline or betaine. Similarly glycerol was not detected.

The evaporated, aqueous filtrates from the lead acetate precipitation were examined with the following results.

1. Very little phosphorus was present.
2. Negligible traces of fatty acids and no glycerol were found.
3. Choline was detected only microchemically as the crystalline periodide. Having due regard to the sensitiveness of this reagent and the large volume of extract from which this amount of choline had been obtained, this choline provides no evidence of the presence of a phosphatide but probably diffused as such from the cell sap.
4. The evaporated aqueous residues consisted of a thick syrup, which contained sugar, was very deliquescent, and, though readily soluble in water and moist alcohol, was insoluble in all dry organic solvents. This was obtained in a relatively large amount, and does not appear to be mentioned by Cranner or Grafe and Horvat [1925].

It is quite clear that the results obtained by leaching potato tissue do not confirm those of Cranner. The experiments of Grafe, however, were carried out with sugar beet tissue. In order to enable a strict comparison between our results and those of Grafe and Horvat [1925], leaching extracts from sugar beets were examined. Two methods of leaching were adopted. It was obvious that in the earlier experiments much of the turbidity of the solutions was due to bacterial activity, plate cultures of turbid extracts always revealing extensive contamination. Moreover, sterile leaching experiments on a small scale indicated that at room temperature the leaching liquid remained clear indefinitely, provided the conditions were aseptic. Hence the following method of leaching was adopted to supplement that of Cranner, and Grafe and Horvat.

The tissue, arranged in layers, was separated by "grids" of bent glass rod which fitted over the legs of an inverted glass tripod, contained in the leaching vessel, which consisted of an inverted bell jar with a gas-tight lid. Tubulures in the bell jar and lid were fitted with ground glass stoppers and tops. Air entering the apparatus did so through cotton wool plugs. Connections were made so that communication could be made at will with three storage vessels containing (1) boiled distilled water cooled out of contact with air, (2) alcoholic mercuric chloride solution, and (3) 0·25 % formaldehyde

solution. The apparatus was sterilised by being completely filled with alcoholic mercuric chloride, washed with boiled distilled water, and partially filled with 0·25 % formaldehyde solution. The tissue was quickly inserted "en bloc," the apparatus then being completely filled with 0·25 % formaldehyde, and emptied by siphoning back into the storage vessel by means of the water-pump. After thorough washing with boiled distilled water, leaching was allowed to proceed with daily changes of distilled water. This procedure reduced bacterial growth to a minimum, especially over the short periods (4 or 5 days) during which one sample of tissue was in use. It is to be noted, however, that the temperature of leaching was about 18° and since all air was excluded by recently boiled water the conditions were approximately anaerobic. Extracts were also collected by the method of Cranner [1922] and Grafe and Horvat [1925], namely, immersion of well washed slices of tissue in single layers in distilled water contained in shallow trays at 20° for 24 hours. After this period the tissue and water were changed. The shallow tray method assures more efficient aeration, but involves greater bacterial contamination.

Utilising this leaching technique, extracts were collected from both red and sugar beetroot slices. These are entirely confirmatory and only the results with sugar beet will be described. A further check experiment, in which 150 g. of the dried lead acetate precipitate were obtained by leaching in a large glass tank, also corroborated the data given below. The method of lead acetate precipitation was adopted for critical purposes only, for it is not one which is usually employed in the isolation of phosphatides. The results obtained were verified by extraction of the aqueous leaching extract with ether. Using the same concentration of normal lead acetate adopted by Grafe and Horvat, the precipitation was effected directly without the preliminary concentration at 28° practised by these authors. In all cases both the lead acetate precipitate and filtrates were examined for the decomposition products of phosphatides.

*Examination of products for phosphatides.*

The hydrolytic agent applied to the washed, dried lead acetate precipitate was 5 % sulphuric acid, allowance being made for the lead content of the precipitate. It is clear that the system of fractionation utilised by Cranner [1925] had not justified itself and it was not adopted either in these experiments or those of Grafe. The hydrolysis was effected by boiling under a reflux condenser for 6 hours. During this period carbon dioxide was evolved and the supernatant liquid became dark coloured. The residual lead sulphate, after being dried and extracted in a Soxhlet apparatus with alcohol and ether for fatty acids, gave an estimate of the lead content of the precipitate correct to about 1 %. The contents of the alcohol and ether extracts of the lead sulphate were united to the ether extract of the acid filtrate, and the saponifiable and unsaponifiable contents determined approximately by the usual

methods. Bases were precipitated from the ether-extracted filtrate, after hydrolysis, by means of phosphotungstic acid, also in 5 % sulphuric acid. Standard methods were utilised in the hydrolysis and fractionation of the phosphotungstates obtained [see Barger, 1914; Guggenheim, 1924]. These removed bases other than those of the simple choline or betaine type. Figures typical of several sets obtained are given in Table I, and for comparison those calculated from the formula of Grafe and Horvat for the lead acetate precipitate are cited.

Table I.

| Leaching method | Weight of ppt. g. | $PbSO_4$ g. | % Pb. | Fatty acid: Weight in g. | Fatty acid: % | Weight of phosphotungstates g. | % Choline | % Glycerophosphoric acid |
|---|---|---|---|---|---|---|---|---|
| Approx. "sterile" | 55 | 61 | 75·2 | 0·50 | 1 | 0·37 | * | * |
| "Shallow" tray | 20 | 21 | 71·2 | 0·12 | 0·6 | 1·0 | * | * |
| Grafe and Horvat | — | — | 36·10 | — | 47·0 | — | 5·3 | 15 |

* None detected.

The complete failure to detect choline and glycerophosphoric acid or glycerol, as hydrolytic products of the lead acetate precipitate, and the remarkably small amount of total fatty acids obtained as compared with that obtained by Grafe and Horvat, is a confirmation of the previous results on potato tissue.

The following analyses given in Table II seem to suggest that the bulk of the lead acetate precipitate is inorganic in nature.

Table II.

| Leaching method | % N | % $CO_2$ | % Pb | % P |
|---|---|---|---|---|
| "Approx. sterile" | 0·115 | 8·26 | 75·2 | 0·42 |
| "Shallow tray" | 0·47 | 9·3 | 71·2 | — |

Nitrogen estimated by Kjeldahl method: carbon dioxide in a modified Schrötter flask; phosphorus by the Pemberton-Neumann method.

Since lead and $CO_3$ account for 86 % of the total precipitate, not more than 10–13 % can be organic. Moreover, of the phosphorus content a considerable percentage is inorganically combined. The liquid obtained by leaching 40 g. of beetroot, in the form of discs, in 100 cc. of distilled water was analysed for phosphorus by the method of Stewart and Archibald [1925]. It was found that 25 cc. contained 0·000232 g. of phosphorus of which 0·000105 g. (or about 45 %) was inorganically combined. This would appear as lead phosphate in the precipitate obtained with normal lead acetate.

It would appear then, that the precipitate obtained by adding normal lead acetate to the aqueous extracts of living tissue collected by the two methods described does not contain appreciable amounts of phosphatide. This is in harmony with our previous results on potato tissue and contrary to Grafe and Horvat, whose procedure has been duplicated in the shallow tray method at 20°. The results given indicate that the molecular formula

derived by Grafe and Horvat cannot represent even an appreciable fraction of the precipitate examined. Moreover, the widespread use of lead acetate, as a general precipitant for various plant products, would indicate that the isolation of a pure chemical compound by its application to a liquid, which probably represents a very dilute solution of the more diffusible cell contents, is extremely improbable. The obscurity in the attachment of the four lead atoms also detracts from the value of this formula. Whilst the substance is described as a single chemical compound of constant composition, the lead atoms (amounting to 36 % of its weight) are stated to be "physically combined" [Grafe and Horvat, 1925, p. 464]. It is true that the preliminary concentration at 28° utilised by Grafe and Horvat may have decreased somewhat the carbon dioxide content of the solutions precipitated, but in view of the low temperature of evaporation, which is little higher than the leaching temperature and lower than that adopted by Cranner (30°), this will not materially effect the criticism that in our experiments the precipitate examined did not contain phosphatides, but consisted mainly of inorganic salts, with a small percentage of fatty acids and amino-acids and probably protein decomposition products. It is probable that a small organic residue, which is not fatty in nature, remains undescribed.

*Examination of the filtrates from lead acetate precipitation.*

Since no evidence of phosphatides could be detected in the precipitate it became of importance to examine the filtrate for phosphatides or their decomposition products.

The accumulated, concentrated filtrates from lead acetate precipitation of leaching extracts from sugar-beet were examined for fatty acids, bases and glycerol. Excess of lead was removed by sulphuric acid and the slightly acid, filtered liquid extracted with ether. Only negligible traces of ether-soluble material were obtained. Bases were precipitated in 5 % sulphuric acid by phosphotungstic acid by the usual method, and the bases regenerated and fractionated by the Schultze method for separation of choline and betaine. Since betaine is a diffusible constituent of the cell sap, it was obtained in appreciable quantity. Of the crystalline hydrochloride (M.P. 227° with decomp.) 0·2 g. was obtained in a state of purity and the crystalline gold salt (M.P. 240°) and picrate (M.P. 180°) prepared. The derivatives agreed in general properties with the respective betaine derivatives. The choline fraction was comparatively small. An alcoholic solution containing a hydrochloride, which yielded a crystalline gold salt, not due to betaine, and also gave the Florence crystal reaction attributed to choline, was obtained. As only microchemical reactions could be applied, it was not possible to state with certainty that choline was present, although this is probable.

After the separation of bases as phosphotungstates, excess of sulphuric and phosphotungstic acids were removed by baryta solution and excess of barium as barium carbonate. The concentrated filtrates were tested unsuccess-

fully for glycerol, but revealed the presence in relatively large amount of the deliquescent syrup, previously noticed in the experiments on potato tissue. In bulk, this appears to be the principal constituent of such leaching extracts, and, whilst some sugar is undoubtedly present, a fraction could be obtained, by repeated precipitation from dilute alcohol by acetone, in a form almost free from nitrogen, phosphorus and reducing sugar, being very soluble in water or moist alcohol, insoluble in all dry solvents and not yielding any crystalline or volatile derivative. No conclusion as to its nature can be drawn, but a somewhat similar product is described by Szent-Györgyi [1925] and has also been examined by Platt and Wormall [1926]. The previous investigators of leaching extracts do not describe this material.

The examination of the filtrates from lead acetate precipitation gives no indication that the leaching extract contained phosphatides, or even fatty acids, which are not precipitated by lead acetate.

In order to test the possibility that Grafe and Horvat might have obtained their results from tissue whose semi-permeability had been impaired, 19 g. of dried lead acetate precipitate, obtained by precipitating the solution containing the products leached from beetroot into water containing chloroform, were hydrolysed. Whilst this showed a closer approximation to Grafe's figures, the total amount of fatty materials was still much below that required by Grafe's data (see Table III and compare Tables I and II).

Table III.

| | |
|---|---|
| Weight of precipitate hydrolysed | = 19 g. |
| Total material soluble in alcohol or ether | = 2·5 g. or 13·1 % |
| Ether-soluble acid after saponification | = 1·1 g. or 5·2 % |
| Unsaponifiable material soluble in ether | Nil |
| Ether- and water-insoluble acid after saponification | = 0·33 g. or 1·7 % |
| Lead sulphate | = 11·4 g. or 40 % Pb. |
| Phosphorus | = 0·87 % |

An attempt to isolate phosphatides from potato tissue by extraction methods will now be described. The procedure was as follows.

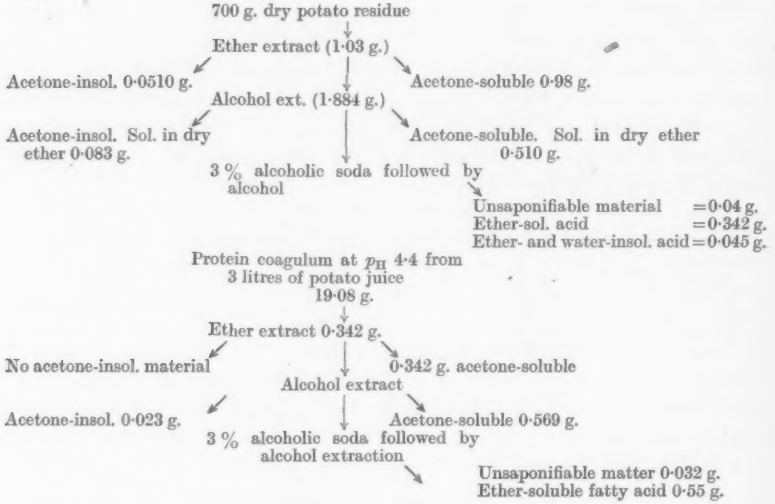
1. Whole tubers, free from the cork layer of the skin, were finely minced.

2. Juice was pressed out in a hand press, the residual material being grey-white and in the form of a hard cake.

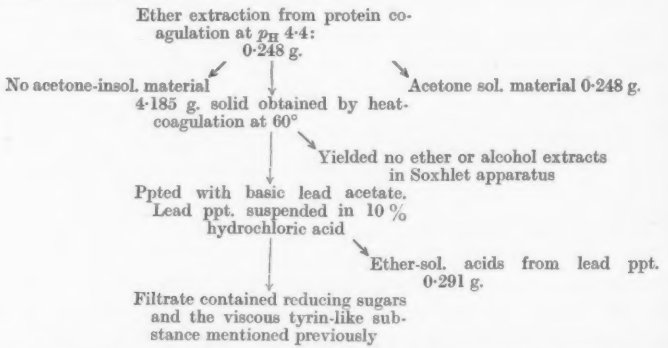
3. The solid residue was air-dried and finally ground to a fine powder in a coffee mill and sieved through a fine mesh wire gauze. 700 g. were then obtained for extraction in a large Soxhlet apparatus.

4. Direct ether extraction of the aqueous liquid (3 litres) was not practicable owing to its opalescent, colloidal nature, so that the reaction was adjusted electrometrically with hydrochloric acid to $p_H = 4{\cdot}4$. After standing, centrifuging and decanting, a yellow-brown solution and a coagulum, which could be examined separately, were obtained. The coagulum was dried in a vacuum desiccator and ground in a mortar and was thus suitable for direct extraction with fat solvents. The products obtained from the dry potato

residue, the protein coagulum and the clear aqueous liquid are described below.


700 g. dry potato residue
Ether extract (1·03 g.)
Acetone-insol. 0·0510 g.
Acetone-soluble 0·98 g.
Alcohol ext. (1·884 g.)
Acetone-insol. Sol. in dry ether 0·083 g.
Acetone-soluble. Sol. in dry ether 0·510 g.
3 % alcoholic soda followed by alcohol
Unsaponifiable material =0·04 g.
Ether-sol. acid =0·342 g.
Ether- and water-insol. acid =0·045 g.
Protein coagulum at pH 4·4 from 3 litres of potato juice 19·08 g.
Ether extract 0·342 g.
No acetone-insol. material
0·342 g. acetone-soluble
Alcohol extract
Acetone-insol. 0·023 g.
Acetone-soluble 0·569 g.
3 % alcoholic soda followed by alcohol extraction
Unsaponifiable matter 0·032 g.
Ether-soluble fatty acid 0·55 g.

The residual liquid was treated as follows:


Ether extraction from protein coagulation at pH 4·4: 0·248 g.
No acetone-insol. material
Acetone sol. material 0·248 g.
4·185 g. solid obtained by heat-coagulation at 60°
Yielded no ether or alcohol extracts in Soxhlet apparatus
Ppted with basic lead acetate. Lead ppt. suspended in 10 % hydrochloric acid
Ether-sol. acids from lead ppt. 0·291 g.
Filtrate contained reducing sugars and the viscous tyrin-like substance mentioned previously

The only fractions which, according to normal technique, would be regarded as crude phosphatide are the acetone-insoluble but ether-soluble ones, amounting in all, from 4 kg. of potato tissue, to 0·16 g. It is also significant that 0·14 g. of this was obtained from the dry residue and not from the aqueous suspension. The materials in question gave qualitative phosphorus reactions, but were not light-sensitive. Whether or not they really contained phosphatides it was impossible to decide with the available material.

In the light of these results obtained by extraction of potato tissue with fat solvents, the following previous observations are of interest.

1. Lippmann [1887] extracted sugar beet with alcohol and ether and was able to isolate lecithin, hydrolyse it and identify the decomposition

products—oleic acid, glycerol, phosphoric acid and betaine. A subsequent experiment gave choline and no betaine.

2. Gallagher [1923], who thought that lecithin could function as an autoxidisable substance in connection with the potato oxidase system, extracted 3 kg. of finely minced tubers with 5 litres of 90 % alcohol. The evaporated solution yielded 1·6 g. of substance soluble in ether, dehydrated by sodium sulphate, and insoluble in acetone. This contained phosphorus, but not choline, and no mention is made of any isolation of fatty acids or glycerol. This also indicates that the amount of lecithin is at any rate small, although the figure for ether-soluble, acetone-insoluble material is somewhat higher than that already given. The effects of variety may be important in this respect, and also the time of extraction relative to the rest period. The tubers used in the author's experiments had been stored during the winter, and were analysed in June.

3. Chibnall and Channon [1927] were unable to isolate lecithin in experiments involving the extraction of some 200 kg. of cabbage leaves. The substance isolated, of a phospholipin character, proved to be in the form of the insoluble calcium salts of glyceridephosphoric acids—in effect lecithin with choline replaced by calcium—and the possibility of true lecithin being present was negatived.

SUMMARY.

Experiments with various parenchymatous tissues lead to the conclusion that leaching experiments do not yield evidence that phosphatides are present in the surface layer of the protoplasm, and diffuse from living tissues into distilled water.

The author desires to acknowledge his indebtedness to Professor J. H. Priestley, in whose laboratory this work was carried out, for his continued help and advice, and also to the Department of Scientific and Industrial Research for a grant of maintenance.

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# XXXVIII. ANTINEURITIC YEAST CONCENTRATES. III.

## THE CURATIVE PIGEON TEST: A CRITIQUE.

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### PART I.

#### Standardisation of the test.

Curative tests upon the pigeon have two main advantages over other methods of testing for the antineuritic principle. Firstly, it is not yet absolutely certain that rat tests can differentiate the curative factor in the vitamin B complex. This matter has been discussed elsewhere [Kinnersley and Peters, 1927], so that it is not necessary to reiterate the evidence here. Secondly, the pigeon is an easy bird to obtain, and to manage, and further it gives a rapid answer with small amounts of test material. It has been the object of the tests carried out in this Department during the last four years to discover the errors to which the test is liable and to learn how to avoid them. An account of the position of the pigeon test is needed at the present time, owing to the appearance of statements in the recent literature tending to discredit the use of the pigeon for short curative experiments. Jansen and Donath [1927] for instance discarded the pigeon as a test object owing to confirmation of Theiler's experiment, in which it was found that birds could be occasionally cured by injections of distilled water. Eijkman [1927] lays great stress upon the necessity of prolonged tests upon the pigeon. In a recent paper by Plimmer, Rosedale, Raymond and Lowndes [1927] the authors have used both curative and preventive tests. They have found that the results obtained with the curative test depend upon the state of the bird, and that, though there is general agreement between the preventive and curative tests, occasionally there are wide divergences. These discrepancies may be due to some inherent error in the curative tests; they may however be ascribed to the fact that preventive tests carried out over prolonged periods such as 60–100 days test for more than one factor. This latter explanation would seem to be the conclusion to be drawn from the results recorded in this communication.

In an earlier paper by one of us [Peters, 1924], certain conditions were enumerated as essential for the success of the curative tests. These were briefly as follows. Only the birds which show the classical symptoms of head retraction should be used. Of these only those showing the symptoms before 30 days can be relied upon, and then only if they seem to be strong enough to stand the test. It was further emphasised that unless a cure was effective for more than 24 hours, it must be ascribed to some temporary effect. An example of such a temporary cure was described, in which the administration of distilled water (by the mouth) cured for a few hours. The test as described in the early paper was used in working out a method of preparing concentrates from yeast of an activity of about 3 mg. per day. In spite of the successful use of the test in preparing these concentrates, other results appeared of a puzzling nature. It was found for instance that small doses of histamine would often produce genuine cures (5 out of 18 birds treated). That such an effect was not due to the presence of minute traces of adsorbed vitamin in the histamine samples was shown by proving that the onset of symptoms was not affected by giving daily doses of histamine. Thus histamine could not function as a protective substance though it could occasionally cure. This confirmed certain earlier conclusions of Abderhalden [1923, 1]. Among a number of other substances tried, a cure was produced by olive oil. Other points brought forward in the same paper were the importance of avoiding cold shock, and the value of keeping the pigeons in the early stages of feeding in a relatively cold place. In a subsequent paper [Kinnersley and Peters, 1925] the technique was slightly modified. The birds were kept in a cool room until the symptoms had either begun to develop or were threatening. They were subsequently transferred as soon as possible to a warm room, and left there for a matter of 2 hours at least before commencing the tests. It was found that such birds would often clear up for 1–3 days, the condition being known as "heat cure." This technique has not been varied significantly since then except in regard to the administration of glucose, to which reference will be made below.

The curative test has been used quantitatively by noting the number of days after cure for which the bird is protected from a second appearance of symptoms. It has been tacitly assumed that only one factor is concerned in producing the cure and the subsequent protection. This is only likely to be true where comparatively short periods of experiment are concerned. As a general rule, the test has been sufficiently quantitative to cause no difficulty in following the distribution of activity during a fractionation. However, as experience of the birds has increased, it has become clear that there are always some abnormal cases. Occasionally this abnormality has become sufficiently serious to disturb the tests. One such abnormal case was shown in Table I [Kinnersley and Peters, 1925]. Whereas the average birds reacted to doses of the preparation of between 0·068 and 0·11 mg., the abnormal case only required 0·025 mg. of the preparation *per diem*. These cases therefore

require much less than an average dose to cure and protect from a further onset of symptoms. In view of this type of abnormality, it is important to enquire whether the quantitative nature of the test is a mere illusion. Especially pertinent is this question in view of the cases of so-called spontaneous cure described by Kon and Drummond [1927], though these have been shown by Kon [1927] not to occur upon rice diets. If the question is considered theoretically, it might be conceived that any process which leads to the cure of a bird will free that bird from further symptoms for an arbitrary period of 2–7 days. In this case the number of days of protection would have only a pseudoquantitative nature, and would be actually due to some random selection and averaging of favourable cases. It is believed by the authors that such a view is incorrect, though it is difficult to obtain a rigid proof of the question owing to the accidental happenings to which the test is liable (such as unobserved vomiting of the dose upon the part of the bird). Three classes of evidence may be adduced in favour of a direct quantitative relation between the dose and the time of protection after cure.

(1) The test has enabled us to work out methods of concentrating the factor some 20,000 times, as compared with pressed baker's yeast (1·0 g. to < 0·05 mg. dry dose).

(2) The administration of a second test dose following upon onset of symptoms after a first dose reproduces the results of the first dose.

(3) The distribution of the curative substance can often be followed quantitatively in a fractionation.

(1) needs no further comment.

(2) In Table I there has been collected a number of instances in which it has been possible to administer a second dose of the same extract as was used for the first dose immediately upon a second appearance of symptoms. Unless such a dose is given soon after the reappearance of symptoms, no conclusions can properly be drawn from the results owing to the ease with which the bird passes beyond recovery.

Table I. *Successive dosing.*

(A) Cases in which the second dose was the same in amount as the first.

| Bird | Protection for: First period | Second period |
|---|---|---|
| 428 | 5 days | 5 days |
| 649 | 3 „ | 3 „ |
| 544 | 3 „ | 3 „ |
| 549 | 5 „ | 3+ „ |
| 790 | 3 „ | 5 „ |
| 709 | 3 „ | 4 „ |
| 587 | 3 „ | 2 „ |
| 713 | 3 „ | 4–5 „ |

(B) Cases in which the second dose was double in amount.

| | | |
|---|---|---|
| 581 | 2 days | 4 days |
| 908 | 3 „ | 6 „ |

N.B. + means that the bird was cleared up with marmite before head retraction had occurred again.

In each of these cases, except No. 649, the symptoms were those of head retraction. In No. 649 they were the so-called forward symptoms (McCarrison's emprosthotonus). In cases (B) twice the dose was given upon the second occasion. These experiments suggest that an individual bird reacts in a standard way to a given preparation. The (B) cases are especially interesting in so far as they show that the number of days' cure for a given bird can be varied by varying the dose. The results of Table I are difficult to explain upon a theory of random sampling.

(3) Many examples have been obtained in the course of the work in which, during a fractionation, the final doses obtained from precipitates and filtrates have added up to a value very close to the original number. Space does not admit the giving of these in detail, but two instances may be quoted. In a silver fractionation upon material of original activity 1·2 mg., 425 doses could be accounted for out of an original 450. Again in the course of a charcoal fractionation 710 doses were recovered out of 720 in the original. These favourable results are not always obtained, a matter which will be discussed below, but the fact that they can be produced upon occasion suggests strongly the underlying quantitative character of the response.

Taken collectively, the evidence above is enough to show that pigeons test for some definite substance in a quantitative manner.

*Certain difficulties.* Accepting the view however that there is fundamentally a relation between the amount of the factor supplied and the time of protection after cure, there is no doubt that a proportion of the birds behave abnormally. Excluding cases due to obvious experimental error (such as improper administration of the dose), some birds in each batch and occasionally many in a given batch behave irregularly in the sense that much less of a given preparation is needed to cure and protect them than the average. It is clear that unless such cases can be reduced to a minimum or controlled, the test becomes unreliable. We have attempted to explain these abnormalities. At first they were attributed to faulty technique, as it is noticeable that at these times a smaller proportion of the birds develop typical symptoms before the 30th day. Improper cleaning of the cages and consequent feeding upon excreta might lead to this result. However, special attention to these details did not improve the position. One period when it was especially evident was in November and December 1926, when the matter was sufficiently serious to upset the tests. An illustration will perhaps make clearer the nature of the difficulty. During the course of an alcohol fractionation, the results shown in Table II were obtained.

There is a wide variation during the fractionation, from 600–1870 plus. "Plus" represents the fact that the bird was cleared upon the 10th or 11th day by marmite before it had shown symptoms again. After 10 days results are not reliable. It might at first be thought that these results could be explained by a difference in the weight of the birds. Reference to Table II will show that this is not so. It might be thought also that a bird which

showed symptoms earlier needed more of the active principle than one maturing later upon a rice diet. With the possible exception of 599, this does not seem to be true.

Table II. *Alcohol fractionation. Case of bad variation in tests.*

During the successive removal of precipitates, the following bird results were obtained. (*a*), (*b*), etc. means result obtained at successive stages.

| Stage | Bird | Result in day doses | Weight of bird at onset of symptoms | Day of symptoms |
|---|---|---|---|---|
| (*a*) | 286 | 750 | 285 g. | 25th |
| (*b*) | 532 | 1700+ | 205 | 24th |
| (*c*) | 501 | 1870+ | 300 | 28th |
| | 599 | 600 | 285 | 10th |
| (*d*) | 606 | 1000+ | 235 | 24th |

Upon a reference to the books it became clear that the birds which gave such a large proportion of abnormal results came from a batch which had been placed upon the polished rice diet immediately upon receipt from the dealers. Out of a batch of 30 birds, only 12 were obtained with the correct symptoms for treatment, and of these 12 at least 8 gave abnormally high answers. This suggested that some storage owing to a previous period of feeding was influencing the results. Such storage effects are already very familiar to workers upon the fat-soluble vitamin. The matter was tested directly by comparing the behaviour of two batches of birds under exactly comparable conditions, as regards temperature, feeding and exercise. One batch of 8 birds (A) was placed upon the polished rice diet upon receipt from the dealer, the other (B) of 11 birds was standardised before polished rice feeding upon a mixed diet in the laboratory for a month. A summary of the results obtained is given in Table III.

Table III.

(A) 8 birds. Placed on polished rice diet immediately upon receipt from the dealer.

| Bird No. | Colour | Weight when symptoms appeared g. | Day of symptoms | Days' cure and protection |
|---|---|---|---|---|
| 787 | Grizzle | 215 | 16th | 8 |
| 790 | Blue bar | 245 | 23rd | 3, 5* |
| 789 | ,, | 260 | 27th | 5 |
| 704 | Grizzle | 230 | 29th | 5 |
| [794 | Blue chequer | 260 | 31st | (12)] |

No symptoms were shown by the 38th day by three other birds, one of which was blue and the others red.

(B) 11 birds. Placed on polished rice diet after previous period of laboratory feeding.

| | | | | |
|---|---|---|---|---|
| 428 | Blue bar | 310 | 16th | 2 |
| 722 | White | 235 | 16th | 5 |
| 709 | Mealy | 225 | 20th | 3, 4* |
| 549 | Blue chequer | 275 | 21st | 7 |
| 684 | Blue chequer pied | 285 | 21st | 4 |
| ? | Grizzle | — | 23rd | No test |
| 587 | Blue chequer | 285 | 23rd | 3, 2* |
| 649 | Mealy | 230 | 27th | 2 |
| 713 | White | 230 | 28th | 3, 4½* |

No symptoms shown by two other birds by 38th day. One of these was a blue bar, and the other a grizzle.

* Indicates that a second dose following the first gave the second figure.

As each case developed, the bird was given a dose of 0·2 cc. of a standard curative preparation. Comparing the behaviour of the two batches, a larger proportion of those in Batch B showed symptoms before the 30th day, 81 % in B against 63 % in A. Of the birds received fresh from the dealers 3 never showed symptoms at all. Also out of the 5 cases from Batch A, 2 gave abnormally high responses, namely, 787 and 794. It is not a question of the day upon which symptoms appeared, because one showed these upon the 15th day whereas the other did not show anything until the 31st day. Of the cases obtained from Batch B, only one case, namely 549, gave an unreasonable response as compared with the others. This proves that the previous feeding influences the effects produced by a period of rice feeding. Further conclusions may be drawn from these results. There is no correlation between the colour of the bird and behaviour to the diet or to the test dose. This reversed a previous impression which we had obtained that the white bird was more sensitive. Also there seems to be no correlation between time of onset of symptoms, length of protection after cure and weight of birds at the onset of symptoms.

If we confine ourselves to Batch B, it will be seen that 9 of the tests gave responses between 2 and 4 days, and that 2 others fell outside these limits at 5 and 7 days.

Taken apart from the evidence in the earlier part of the paper, these results might be construed to mean that any agent which cures, does so for a period merely depending upon the bird and that there was no true quantitative happening. If we confine ourselves to the results of Batch B alone, there seems to be a better case for quantity. In one respect these results were not satisfactory. The stock solution used for dosage was found subsequently to contain rather large amounts of metals, which we have found to be apt to cause irregular results. The matter was left until the summer vacation when the opportunity offered for reinvestigation. It was decided to test again the influence of standard feeding upon the number of cases obtained in the 30-day period; and further to see whether, with standardised birds, more standard responses to a given test dose could be obtained with the same preparation as that used in the experiments of Table III, but after it had been freed from metals. The stock birds were therefore fed for 5 weeks upon the mixed laboratory diet, and subsequently placed upon polished rice. The results were rather surprising. Practically all birds placed upon the polished rice developed the characteristic symptoms before the 30th day, in some batches of 20 every bird became a case for experiment. This proved that birds before test should be fed in a standard way.

Even among these birds the abnormal case still appeared, though less frequently. Before continuing with the description of the further experiments with the stock preparation, it is necessary to describe a further improvement in technique, which has been introduced with a view to eliminating the abnormal case. In the hope of explaining some of the abnormal responses

obtained, especially with preparations of under 0·1 mg. per day activity, small doses of glucose were given with some of the preparations. This led to a test of the effect of small doses of glucose (20–50 mg.) given alone. To our surprise these doses seemed to be capable of curing certain birds. The glucose sample had been prepared by one of us (V. R.) by purification with charcoal and subsequent recrystallisation from 75 % alcohol. It contained no trace of the *Streptothrix* growth factor[1], and it is inconceivable that the effect could be due to adherent vitamin. An amount of 50 mg. may seem to be too small to have food value, until it is remembered that the weight of these birds is not more than 250 g. If the weight of the blood be placed as high as one-twelfth of the body weight and the blood-sugar at 0·2 %, then the amount of sugar in the blood of one of these pigeons would be 40 mg. In most cases, like the "heat" cures, the "glucose" cures do not persist for long, and cannot be repeated by giving a further dose. In two cases it has been found that doses of glucose would cure a bird even for a second time. Table IV gives the details of these two cases.

Table IV. *Glucose.*

*Case A.* Bird 981. Blue chequer pied. Developed symptoms upon 23rd day of feeding, and was given 20 mg. glucose. Bird cleared up for 7 days and was then given a further 20 mg. glucose which again cleared it up for two more days. Upon the third occasion glucose had no effect and the bird died.

*Case B.* Bird 60. Blue chequer. Brought into warm room with threatening convulsions upon 22nd day of feeding. Upon 23rd had head retraction, and was given 20 mg. glucose which cured in 4 hours. The cure lasted until 25th day, when more glucose was given. This cured the bird until the 26th day, when it was used for experiment. In this case it is certain that temperature played no part in the results.

It is proposed to return to a discussion of the "glucose" cure later. For the purpose of the further experiments upon the stock preparation it is sufficient to note that the further precaution was taken of giving all the test birds a dose of purified glucose as well as allowing ample time for the development of any possible heat cure, before giving the test dose.

Table V.

Preparation. Stock H. 0·1 cc. = 1/1900 of the whole.

| Dose | Bird | Day on which symptoms appeared | Days of cure and protection |
|---|---|---|---|
| 0·1 cc. | 37 | 23rd | 2 |
| | 52 | 24th | 2 |
| | [60 | 24th | (6)?] abnormal |
| | 2 | 31st | 2 |
| 0·2 cc. | 30 | — | 3 |
| | 1 | 23rd | 4 |
| | 56 | 30th | 4 |
| | 98 | 18th | 7 |
| | 66 | 28th | 7 |

Table V gives the results of tests carried out upon birds standardised by a period of feeding for at least a month upon the mixed laboratory diet. The questions to be answered were; firstly, whether birds selected at random for test behaved in a standard way to a given test dose; secondly, what was

[1] This work is being prepared for publication.

likely to be the limits of variation in the number of days of protection after cure for a given dose; and, thirdly, whether increase of the dose increased the number of days of protection after cure proportionately. It had been our impression that a small dose upon the margin of the curative one (2 days and under) gave relatively a low result as compared with a higher dose.

The results are in general less variable than those of Table III. The abnormal case has been included in the table because of its interest. It stamped itself as abnormal by the fact that the bird was twice cleared up by doses of glucose. Excluding this case, which would be automatically excluded by the present methods of testing, the results are, for a dose of 0·1 cc. 2 days, for 0·2 cc. 5 days $\pm$ 2. These seem to be the best results which we can obtain for pigeons selected at random for the tests. For larger doses seè Kinnersley and Peters [1925, p. 821]. In answer to the third question, it is seen that increase of the dose has increased the number of days of protection after cure, though not quite proportionally. If we make the assumption, which is reasonable, that one dose is used in clearing the bird and add one day to each of these figures, there is exact correspondence between the figures, namely, 0·1 cc. 3 days, 0·2 cc. 6 days. The variations of the test from the mean seem to be $\pm$ 40 %. By taking the average of two birds, the chances of wide variation are of course much reduced.

The above experiments appear to establish the fact that there is a quantitative relation between the time of protection after cure and the amount of the curative dose given, provided that short periods of protection are considered, namely, not more than 10 days, and that birds are not allowed to exceed a period of 30 days upon the polished rice diet. The experiments also define the limits under which the test can be used with success.

## PART II.

### The response of the pigeon to a polished rice diet.

The quantitative behaviour of the rice-fed bird to test doses proves that each bird uses *per diem* a certain amount of this factor in the course of its metabolism. This amount appears to be a diminishing one, because animals which survive over the 30th day seem to require only small amounts of the active principle to keep them alive. At this stage the metabolism is shut down. Now the data in Part I show that there is a variation in the quantitative response amounting to some 40 % either way. This suggests that there is some difference in the amount of active principle required by different birds. If this be so, then it should be possible to show that different birds react to a polished rice diet differently. A bird which gives a longer number of days in the tests for the active principle should also take longer to develop symptoms upon the polished rice diet. This has been considered briefly in Part I, but no suggestions upon the matter can be entertained unless it prove to be a fact that birds react constantly to the polished rice diet, so far as the number

of days required to reach symptoms are concerned. Accordingly a search through the accumulated data of the last 3 years has been made (involving some 2000 cases). Records have been found of 63 cases in which birds have been used for tests 3 times or more, in 20 of these the birds having survived

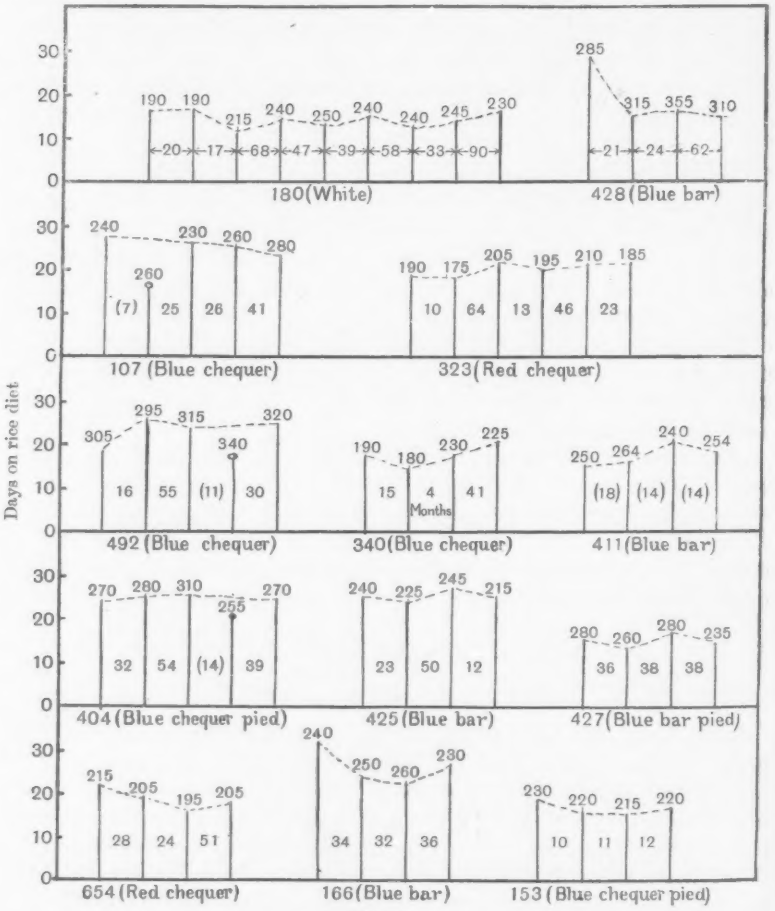


Fig. 1.

A. Vertical lines represent the length of time on rice before developing symptoms.
B. Numbers between the vertical lines represent the number of days interval on normal diet between any two experiments.
C. Numbers at the top of vertical lines show the weight of the bird upon the day upon which symptoms developed.

more than 3 test periods. The data were only collected as an incident in the testing work and in some cases cannot be cited owing to the absence of essential points. There are however enough complete records to deal with

the issue raised. Does an individual bird always develop symptoms on or about the same day after the commencement of rice feeding?

The chart (Fig. 1) gives the essential data for 13 cases in which birds have been used for experiment more than 3 times. The cases in which birds have survived only 3 treatments are given separately in Table VI. Upon the chart are recorded the colour of the birds, the number of days before appearance of symptoms (reckoned from the day upon which the bird was placed upon polished rice), the number of days interval of ordinary feeding between the experimental periods and the weight of the birds in each case at the onset of convulsions. The conclusions from Part I suggest that birds would react irregularly upon the first period of feeding, because most of the results here recorded were undertaken before recognition of the necessity for a preliminary period of standard feeding. Such an irregularity can be observed in the case of birds 428, 492, 425 and 153 and Table VI. Excluding this first period of feeding, the majority of birds show symptoms of head retraction on or about a day that is rather constant for the bird. For birds 323, 425, 428 and 153, the day for each bird may be regarded as a constant. The most interesting case is that of 180. This survived a period of experiment of more than 18 months, during which there are records of 9 sessions of rice feeding. During this time, the extremes of variation shown by the bird were from 11–17 days, being respectively 16, 17, 11, 14, 12, 15, 12, 14, 16 days. Of these probably 11 should be discarded, because the notes show that the bird was replaced upon the polished rice diet again after the short interval of 17 days upon a normal diet. Several cases of this error have been brought to light during an examination of the notes. In practically all of them the reaction time to the diet has been low and out of harmony with the remaining results. For instance, through some clerical error 107 was replaced upon polished rice after only 7 days of mixed feeding. It gives a response quite out of keeping with the other responses for the same bird. The same effect is seen in 404, 492 and 340. These figures have been excluded from the computation as the bird has evidently not had time to replenish its stores of vitamin. This constancy in the reaction time to a polished rice diet for a given bird was rather unexpected. The point receives strong support from the data upon the three-period cases in Table VI. Out of 41 cases found, 14 have had to be discarded owing to inadequacy of data. The remaining 27 cases appear in the table. It will be seen that 17/27 showed a difference between the second and third responses of not more than one day. Five of the remainder showed deviations of 2 or 3 days. Seeing that responses have varied over a period of 14 to 28 days, it is clear that these correspondences cannot be accidental. Further it is interesting that in the case of 600 and of 634, the high results given in the third figure are out of step with the first and second periods, and oddly enough both came from birds which had been put down upon the same day, April 14, 1927. This suggests that there may have been some error or otherwise in the feeding of this batch.

Table VI. *Three-period cases.*

| No. | Colour | Average weight of last two weighings g. | Periods of feeding (days) First | Second | Third | Differences 2nd and 3rd | Day constant |
|---|---|---|---|---|---|---|---|
| 108 | Red chequer pied | 245 ± 5 | 28 | 19 | 19 | None | 19·0 |
| 217 | Blue chequer pied | 215 ± 5 | 16 | 20 | 20 | None | 20·0 |
| 229 | Blue chequer | 282 ± 2 | 14 | 13 | 15 | 2 days | 14·0 ± 1·0 |
| 286 | Blue chequer | 287 ± 2 | 14 | 24 | 27 | 3 days | 25·5 ± 1·5 |
| 339 | Blue chequer | 225 ± 10 | 23 | 28 | 27 | 1 day | 27·5 ± 0·5 |
| 344 | Red chequer | 340 ± 10 | 16 | 16 | 15 | 1 day | 15·5 ± 0·5 |
| 372 | Blue chequer | 278 ± 18 | 30 | 23 | 24 | 1 day | 23·5 ± 0·5 |
| 409 | Blue bar | 275 ± 20 | 17 | 24 | 24 | None | 24·0 |
| 457 | Blue chequer | 265 ± 15 | 16 | 19 | 19 | None | 19·0 |
| 493 | Blue bar | 222 ± 2 | 21 | 23 | 24 | 1 day | 23·5 ± 0·5 |
| 505 | Blue chequer | 265 ± 5 | 29 | 21 | 21 | None | 21·0 |
| 522 | Silver mealy | 215 ± 5 | 25 | 18 | 19 | 1 day | 18·5 ± 0·5 |
| 568 | White | 252 ± 2 | 22 | 17 | 16 | 1 day | 16·5 ± 0·5 |
| 566 | Blue bar pied | 215 ± 20 | 19 | 16 | 12 | 4 days | 14·0 ± 2·0 |
| 600 | Red chequer | 362 ± 12 | 14 | 14 | 21* | 7 days | Nil |
| 634 | Grizzle | 245 ± 15 | 15 | 14 | 18* | 4 days | 16·0 ± 2·0 |
| 651 | Blue chequer pied | 297 ± 2 | 18 | 19 | 19 | None | 19·0 |
| 763 | Silver mealy | 267 ± 2 | 24 | 19 | 26 | 7 days | Nil |
| 684 | Blue chequer pied | 285 | 17 | 20 | 21 | 1 day | 20·5 ± 0·5 |
| 709 | Mealy | 234 ± 6 | 29 | 19 | 22 | 3 days | 20·5 ± 1·5 |
| 722 | White | 245 ± 10 | 23 | 15 | 14 | 1 day | 14·5 ± 0·5 |
| 726 | Blue | 254 ± 2 | 18 | 19 | 23 | 4 days | 21·0 ± 2·0 |
| 741 | Mealy | 300 ± 5 | 21 | 18 | 18 | None | 18·0 |
| 841 | Red chequer | 317 ± 12 | 22 | 24 | 21 | 3 days | 22·5 ± 1·5 |
| 814 | Dark blue chequer | 224 | 24 | 23 | 26 | 3 days | 24·5 ± 1·5 |
| 869 | Blue chequer | 250 | 24 | 18 | 18 | None | 18·0 |
| 473 | White and blue | 300 ± 10 | 26 | 17 | 18 | 1 day | 17·5 ± 0·5 |

* Apr. 14.

The data give the clear-cut answer that the majority of birds react to the diet in a constant manner. Birds such as 166, Fig. 1, are an exception in not conforming to a general rule. This constancy of reaction to the diet is felt to be of fundamental importance. For an individual bird there tends to be a definite time for which that bird can subsist upon the diet and this time differs for different birds. Birds can be classified into "quick reacting" under 17 days, "average" 17–23 days and "slow" over 23 days. Table VII is an abstract of day constants grouped under the colours of the birds.

Table VII.

| Colour | Number of days |
|---|---|
| Blue chequer | 14, 25·5, 27·5, 23·5, 19, 21, 24·5 |
| Blue chequer pied | 20, 19, 20·5 |
| Blue bar | 24, 23·5 |
| Blue bar pied | 16 |
| Red chequer (brown) | 15·5, 14, 21 |
| Silver mealy | 18·5, 23 |
| Grizzle | 16 |
| Mealy | 20·5, 16·5 |
| White | 17·5, 16·5, 14·5 |

There seems here to be a tendency for the colours other than blue to react more quickly, though this is clearly not a general rule. The results for the blue chequer birds show that there is no reason for attributing slow and quick reaction to the colour. There is also clearly no correlation with the

weight[1]. The thesis of Abderhalden [1923, 2] and Grey [1927] that colours other than blue and black are not sufficiently resistant is not supported by this table. We have noted, however, that fancy birds such as the fantails make unsatisfactory cases.

We are now in a position to ask the further question, namely, whether it can be shown that those birds which react quickly to the polished rice diet give lower values for the test than others. It will be recalled that earlier in this communication it was stated that there was no correlation between the length of time of protection after cure and the day upon which the birds showed symptoms. Reliable evidence upon this point is somewhat scanty; the most reliable, which we have, would seem to be that included in Tables III and V. In Table III (B), the majority of the birds were reacting well to their "day constant." Yet the days of protection and cure do not correspond with the "day constants." This might be attributed to some toxic effect of the metals in the preparation, some birds being more susceptible to inhibitory influences than others. However, this objection is not present in the results of Table V which were obtained upon birds that had been previously standardised by the new technique. Here we have two cases both of which give an answer of 7 days to 0·2 cc. of the preparation, yet one of them has shown symptoms upon the 18th and the other upon the 28th day. Undoubtedly more evidence upon this point is needed, but what evidence exists is against the view that the variations in the test responses can be explained by variations in the "day constant." The puzzle raised by this finding will be discussed later. It may be concluded from this part that after a previous standardisation birds react in a constant manner to a polished rice diet. This constancy is not related to the colour or to the weight of the bird, nor does it explain the variations in the response of pigeons to test doses.

### Summary of the variations which may affect the test.

The following is a summary of all the factors which have been found so far to affect the pigeon test, together with the means of avoiding them so far as they are known.

*Gross errors.* The dose may not be introduced completely, owing to constriction in the throat of the bird. The dose may be vomited by the bird. These errors can usually be excluded.

*Errors of feeding.* Birds should be standardised as regards diet before being placed upon polished rice for feeding. It would be doubtless better to have a standard variety of pigeon, but until such is available it is simpler to use birds at random from the open market.

*Possible cures other than that due to the curative substance.* The "heat" cure. This can be avoided by leaving birds in the warm for at least 2 hours before treatment.

[1] Unfortunately, data as to sex and age are not available. Pigeon 180 however suggests that age is not the determining factor.

*The "glucose" cure.* This can be avoided by giving a small dose of glucose in water, at the same time that the bird is being tested for "heat" cure. This also has the advantage that any cure producible by distilled water alone is also eliminated [Peters, 1924, p. 859].

*Note.* The "spontaneous" cure [Kon and Drummond, 1927] has been shown by Kon [1927] not to occur upon a polished rice diet.

It is believed that when these variations are eliminated the number of abnormal cases is reduced to a minimum and is not sufficient to disturb the tests. It is of course tacitly assumed here that the conditions previously laid down are observed, such as the administration of the test dose within 6–12 hours of the onset of symptoms, the elimination of any bird which appears to be too weak for the test, and of all birds which do not show the characteristic symptoms of head retraction within 30 days. A few birds show "forward" symptoms (emprosthotonus of McCarrison [1921]). These we have used occasionally when we have been pressed for test birds, but we have not been able to place much reliance upon the results of the tests. When the sources of error summarised above have been eliminated, we believe that the pigeon "curative" test is rapid, useful and accurate. In the Appendix is described in full the technique used in this Department.

## Discussion.

It is hoped that sufficient evidence has been presented to make it clear that pigeons can be used for curative tests under stated conditions. The most interesting point that emerges for discussion is that birds have an individuality so far as their response to the vitamin-free polished rice diet is concerned. It has been shown that this is not a matter of colour or weight. The explanation must therefore be sought elsewhere. Not much information has been found in the literature upon this matter. With reference to fowls, Eijkman and van Hoogenhuize [1916] state that one of the factors which determines the length of the incubation period is the body weight at the beginning of the experiment. Vedder [1918], in confirmation of earlier work, concluded that the onset of polyneuritis in the fowl bore no relation to the amount of food eaten, but was a matter of the idiosyncrasy of the animal. The view that there is no relation between the time of onset and the amount of the food eaten is not generally accepted [see Braddon and Cooper, 1914; Funk, 1919; Plimmer, Rosedale, Raymond and Lowndes, 1927; Cowgill and Klotz, 1927]. It seems to be held rather widely that there is a relation between the metabolism and the requirements of the vitamin B complex. Using autolysed yeast as a source of vitamin, Collazo and Funk [1924] state that each pigeon requires a different amount of autolysed yeast to keep it in vitamin equilibrium. Their criterion for equilibrium is the maintenance of a constant weight. An examination of their table (p. 190), shows that the heavier birds ate more, and required only 80 % as much vitamin as the rather lighter birds. Summarising the above, it may be con-

cluded that previous observers have noted idiosyncrasy in the animals, but so far as can be ascertained no one has found that the majority of birds can be brought into a condition in which they react to the diet in a constant number of days. There seems to be no explanation at the moment of the idiosyncrasy. We are all aware of marked differences in man in appetite which are not obviously correlated with size or with the degree of adiposity. With regard to the "day constant" for a given bird, it is natural to assume that this represents a day upon which the vitamin store has become depleted in some sensitive part of the nervous system. If it were due to a daily diminution in the stores of vitamin, then a bird which is "quick reacting" should utilise vitamin faster than one which is "slow reacting." A curative dose should last the latter bird longer than the former one. This has been shown in Part II not to be the case, so far as the evidence is available. It is difficult to be quite certain that in these curative tests due allowance has been made for the possible effect of inhibitory factors. These might operate more powerfully in one bird than in another. But the data suggest that the day constant is not merely determined by the daily loss of the curative substance. Some other variable appears to be concerned. This may be the amount of food eaten by the bird, a matter which it should be possible to determine experimentally.

From the standpoint of the curative test, all that is necessary is to make certain that a bird under test is not liable to curative influences other than that of the test extract. It would be of assistance to have some coordinating theory to cover all the cases of cure, of whatever nature this may be. Attempts have been made, especially by Hess [1921] and Abderhalden [1923, 1], to explain the symptoms as due to some failure in the oxidations of the body. That this explanation cannot be applied to the tissues as a whole has been amply shown by the work of Roche [1925] and of Drummond and Marrian [1926]. The latter could find no difference between the tissues of the normal and avitaminosed pigeon as regards oxygen uptake or power of reducing methylene blue anaerobically. They also showed that the lowering of temperature in avitaminosis in the rat is connected with the lowering of body oxidations, which could be restored to normal by raising the temperature of the body as a whole. This proves that there is no general interference with oxidations. But the symptoms of opisthotonus are likely to be connected with a comparatively small part of the nervous system, changes in the oxidation of which may not show up experimentally when merged in the general metabolism. The most hopeful means of coordinating the data seems to lie in the view that the symptoms appear as the result of some local interference with the cycle of metabolic changes (probably of carbohydrate [Collazo, 1923]). Such interference may be produced by lack of a suitable catalyst. It may on the other hand be produced by failure of circulation, causing lack of substrate in the tissue or interference by accumulation of waste products. Histamine, heat, sugared water may all be means of restoring a failing circulation,

which has led to interference in function even in the presence of an adequate concentration of the active principle.

## Appendix.

### *The technique.*

The pigeons are standardised upon receipt from the dealer by placing for periods of 20 days to a month upon a mixed diet in an open-air cage of dimensions 6 ft. high × 6 ft. × 8 ft. The mixed diet has the following approximate composition, buckwheat 1 part, dari 1 part, split maize 1 part, split wheat 1 part and wheat 2 parts.

The pigeons are transferred in batches of 15 to cages of the dimensions 4 ft. 6 in. high × 2 ft. × 2 ft. These cages are fitted with wire bottoms and are cleaned thoroughly every 3 days to prevent the accumulation of excreta. The birds are then given a diet of polished rice (Patna)[1]. The polished rice is prepared by washing for 3 hours with running water, constantly stirring. It is drained, dried before the fire, and then autoclaved at 120° for 2 hours. The cages stand in the indoor animal house at a temperature of 10–15°. When the birds develop symptoms or appear to be about to do so, they are brought into the warm room, temperature about 21°. Here they are placed in smaller cages. Care is taken that these cages should not come into contact with birds which have not been upon a vitamin-free diet for some time. The birds are given a dose of about 50 mg. of purified glucose in 5 cc. of water and left in the heat for 2–3 hours before treatment in order to eliminate the various sources of extraordinary cure. The doses are introduced into the crop with a pipette fitted with a short length of bicycle-valve tubing.

At the conclusion of the experiment, the bird is given a dose of marmite, and placed in a special cage, where it is kept in the warm for 2 or 3 days until completely recovered. The bird is then restored to the open-air cage for a period of 20 days to a month before being subjected again to the rice diet.

## Summary.

1. The evidence for the quantitative nature of the curative pigeon test has been examined. Reasons are given for the belief that the test can be used with confidence, and the conditions necessary for good results are enumerated.

2. Abnormal cases sometimes appear in which birds require much less than the average dose to cure and protect them from a further onset of symptoms. This is due in part to the influence of the previous period of feeding, and can be largely avoided by standardisation upon a laboratory diet before rice feeding. Such a period of feeding also improves the number of cases showing the characteristic symptoms before the 30th day.

[1] Birds are allowed to feed voluntarily throughout. Lumps of salt are also placed in the cages. Oxford tap water is given for drinking purposes.

3. Some birds have been cured by giving small doses of purified glucose. This "glucose" cure must be distinguished from the "heat" cure. As a routine each case may be given a dose of glucose at the time that it is placed in the hot room. The above procedures tend to eliminate the abnormal cases.

4. Variations of $\pm$ 40 % may be expected when pigeons are selected at random for the tests.

5. It is found that a majority of birds react constantly to the rice feeding, there being a constant day upon which the symptoms tend to appear, reckoned from the beginning of the rice feeding. This is termed the "day constant" for the bird, and may vary with different birds from 14 to 28 days. It is not correlated with the colour of the bird, or the weight at onset of convulsions. Birds can be classified into "quick" reacting and "slow" reacting.

6. Variations in the day constant do not explain the $\pm$ 40 % variations in the responses to test doses.

7. A summary of methods of feeding pigeons in this Department is appended.

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# XXXIX. CHEMICAL CONSTITUTION AND TOXICITY.

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In many of the previous attempts to ascertain whether any definite relationship exists between chemical constitution and toxicity the principal difficulty has lain in the selection of a suitable biological standard. In the following investigation protozoa have been used for the measurement of toxicity. This choice was based upon two considerations: firstly, the uniformity (of biological material) obtainable with protozoa cultivated in a synthetic medium was regarded as valuable; secondly, it was felt that the killing-power towards such a primitive organism afforded a measure of general protoplasmic toxicity and avoided specialised toxic effects. Some preliminary work by McCleland and Peters [1919] on the toxicity of certain arsenic compounds to *Paramecium* afforded an introduction to this investigation. Flury [1921] has, in a similar manner, utilised unicellular organisms as a means of comparing the toxicities of arsenic and other irritant compounds. Apart from these instances, however, there appears to be a general recognition of the usefulness of protozoa in the measurement of pharmacological action.

## Method of experimentation.

*Principle.* The principle of the method adopted has lain in the determination of the concentration of substance to be tested which killed all the organisms in a given amount of culture in a fixed time. The unicellular organism used was *Colpidium colpoda*, a culture (derived from a single organism) of which was in the first place supplied by Prof. R. A. Peters. This organism was cultured in the manner described by Peters [1921], in a simple synthetic medium, consisting of one organic and five inorganic salts; the ammonium glycerophosphate (0·06 %) of Peters's medium was replaced by sodium glycerophosphate (0·05 %) and ammonium chloride (0·01 %) owing to the greater degree of purity obtainable by recrystallisation of the sodium glycerophosphate. The culture was originally believed to be a pure one, but during the whole course of this work there has been present an apparently symbiotic bacterium. The possible influence of the presence of this foreign organism upon the toxicity results will be considered later. The cultures

were used for toxicity purposes some 10 or 12 days after inoculation, when the organisms numbered from 3000 to 5000 per cc. At a later stage of this work a second protozoon, which was identified as *Glaucoma scintillans*[1], was introduced. This organism was cultivated in a similar medium to that in which *Colpidium* was grown and it was used for toxicity determinations in parallel with the latter organism.

The concentration required to kill in a given time was chosen as the measure of toxicity, in preference to the time required to kill at a given concentration, since it furnishes a direct value for toxicity as compared with a relative value. There are, however, other objections to the selection of time required to kill for the measuring of toxicity and the foremost of these is the prolongation of the death process, which itself introduces several undesirable features. Firstly, it magnifies the variations in resistance of individual organisms. In the second place, it allows the interference of possible secondary effects arising from decomposition of the toxic agent and the bringing into play of the protective mechanism by the organism. In the third place, it assumes that the effect of a long exposure to a dilute solution is the same as a short exposure to a more concentrated solution; or, in other words, it is assumed that there is a straight line relationship between time and concentration required to kill. Experiments have shown that this is not the case and it is apparent that there is a limiting dilution at which the toxic agent ceases to exert any detectable effect upon the organism. For these reasons it was decided to adopt an arbitrary time limit and the 3-minute period was chosen as being the shortest time compatible with accurate observation.

The death point of the organisms was regarded as occurring when all movement had ceased. The selection of this criterion was based upon the fact that, whatever the initial cause of the cessation of movement, the organisms were never observed to have resumed movement after having once stopped. Owing to the variety of ways in which death may occur, according to the nature of the toxic agent employed, cessation of movement was regarded as being the most satisfactory standard. Prior to death, the movements of the protozoa grew more and more sluggish and finally ceased. Sometimes there was a period of increased activity and occasionally the organism rotated rapidly about its own axis immediately before death; this was especially noticeable in cases where death was due to acidity or to changes in osmotic pressure. Death was followed ultimately by disintegration of the organism.

*Technique.* The actual toxicity determinations were carried out as follows. Ten drops of the culture were transferred to a clean watch-glass, by means of a Dreyer pipette fitted with rubber teat. The number of organisms per drop showed very good agreement. To the culture were added drops of the toxic solution (of known strength) by means of a similar pipette. During the addition of the solution the watch-glass was gently shaken in order to avoid high local concentration. The effect upon the culture was observed under a

[1] Thanks are due to Dr Helen Goodrich for identifying this organism.

low power microscope (1-inch objective). From the number of drops of toxic solution required to kill the organisms in exactly 3 minutes the concentration of toxic substance was calculated and is referred to hereafter as the toxic concentration. If $x$ be the number of drops which kills in 3 minutes, then the toxic concentration is given by the expression $\frac{fx}{fx+10} \cdot \frac{M}{y}$, where $f$ is a factor used in correcting for the difference in volume of the drops delivered by the two pipettes and $\frac{M}{y}$ is the molecular concentration of toxic agent. In practice it was found simpler to select two pipettes which delivered drops of the same volume, thus obviating the introduction of the factor $f$.

Considerable difficulty was experienced at first in making aqueous solutions of known strength of the large number of arsenic compounds examined. As a rule these were but slightly soluble in water. In the end, the following procedure was adopted. A weighed amount of the toxic compound was dissolved in 2–3 cc. of pure pyridine. A measured volume of the pyridine solution was then pipetted into a known large volume of water, accompanied by vigorous shaking of the flask. In this way various solutions of the toxic agent were made up until one was obtained which killed in 3 minutes. The strength of the pyridine in the solution was never allowed to exceed 0·1 %. Control experiments showed that pyridine in 10 times this strength did not affect the organisms in periods exceeding the 3-minute period normally used. Solutions made in this manner could only be used for toxicity purposes when freshly prepared, for it was found that many of the tervalent arsenic compounds dissolved in pyridine or alcohol displayed a marked fall in toxicity on standing. This fall was quite definite after the solution had stood for 24 hours, and was attributed to the absorption of moisture and oxygen by the solution, with the formation of the corresponding arsinic acid. Thus, diphenylchloroarsine in alcohol became transformed, on standing, into diphenylarsinic acid:

$$(C_6H_5)_2As.Cl + H_2O + O \rightarrow (C_6H_5)_2As.O.OH + HCl.$$

*Standardisation of the method.* Before it was possible to place reliance upon this method of toxicity determination it was important to know what conditions would lead to an apparent variation in the toxicity of the substances examined. For this purpose the toxicities of two standard solutions, nickel chloride and mercuric chloride, were followed upon three growing cultures of *C. colpoda*. Tables I, II and III contain the results. It will be seen that there were variations in the toxicities of both solutions amounting to some 40 %. It will also be seen that there was a definite increase in the resistance of the organisms with increasing age and that after a certain period—12 to 14 days—the cultures became less subject to variation and tended more towards a uniform resistance. This period coincides almost exactly with the period required for the cultures to reach their maximum growth, as found by Peters [1921]. For this reason cultures were taken for toxicity measurements, in the subsequent investigations, as nearly as possible

at the time they had reached their maximum growth. Nevertheless, the varying number of organisms was alone not sufficient to account for the 40 % variation obtained, although, perhaps, the most important factor. Diluting the cultures did not result in any significant change in the amount of toxic agent required to kill the organisms. Furthermore, the number of organisms (3000–5000) per cc. represents a comparatively poor growth, so that the proportion of organisms, by bulk, to the medium was exceedingly small (of the order 1 : 100,000).

Table I.

| Age in days | Temperature ° C. | Toxic concentration $NiCl_2$ | Toxic concentration $HgCl_2$ |
|---|---|---|---|
| 8 | 17·6 | *M*/583 | *M*/27,000 |
| 9 | 17·2 | *M*/627 | *M*/25,000 |
| 10 | 17·2 | *M*/800 | *M*/27,000 |
| 12 | 17·4 | *M*/583 | *M*/17,000 |
| 13 | 15·6 | *M*/538 | *M*/19,500 |
| 15 | 16·1 | *M*/460 | *M*/18,100 |
| 16 | 15·6 | *M*/490 | *M*/13,000 |
| 17 | 17·2 | *M*/490 | *M*/16,200 |
| 19 | 15·8 | *M*/490 | *M*/17,000 |
| 20 | 16·2 | *M*/490 | *M*/18,100 |
| Variation | | 44 % | 41 % |

Table II.

| Age in days | Temperature ° C. | Toxic concentration $NiCl_2$ | Toxic concentration $HgCl_2$ |
|---|---|---|---|
| 6 | 17·2 | *M*/800 | *M*/30,000 |
| 7 | 16·7 | *M*/875 | *M*/27,000 |
| 8 | 17·6 | *M*/875 | *M*/27,000 |
| 9 | 17·2 | *M*/707 | *M*/30,000 |
| 10 | 17·2 | *M*/707 | *M*/27,000 |
| 12 | 17·4 | *M*/490 | *M*/23,000 |
| 13 | 15·6 | *M*/490 | *M*/21,400 |
| 15 | 16·1 | *M*/432 | *M*/19,500 |
| 16 | 15·6 | *M*/432 | *M*/17,000 |
| 17 | 17·2 | *M*/410 | *M*/17,000 |
| 19 | 15·8 | *M*/410 | *M*/17,000 |
| 20 | 16·2 | *M*/432 | *M*/17,000 |
| Variation | | 46 % | 43 % |

Table III.

| Age in days | Temperature ° C. | Toxic concentration $NiCl_2$ | Toxic concentration $HgCl_2$ |
|---|---|---|---|
| 10 | 17·2 | *M*/800 | *M*/27,000 |
| 12 | 17·4 | *M*/583 | *M*/21,400 |
| 13 | 15·6 | *M*/583 | *M*/21,400 |
| 15 | 16·1 | *M*/538 | *M*/22,500 |
| 16 | 15·6 | *M*/538 | *M*/19,000 |
| 17 | 17·2 | *M*/490 | *M*/19,000 |
| 19 | 15·8 | *M*/490 | *M*/19,000 |
| 20 | 16·2 | *M*/460 | *M*/19,000 |
| Variation | | 44 % | 30 % |

*Possible sources of error.*

*Measurement of drops.* The drop method, in the first place, does not give results more accurate than one drop of the solution used for killing. In the case of the two standard solutions used, an error upon this account might have amounted to some 5 %, but was probably less. In the measurement of drops from the capillary pipette there may have been an error of not more than 10 %. There was thus a total possible error of not more than 15 %, in partial explanation of the 40 % variation.

*Variation in temperature.* It was anticipated that variations in temperature would exert some influence upon the apparent toxicity of a solution. Toxicity measurements were therefore carried out, using the two standard toxic solutions, upon cultures at widely different temperatures. Two organic compounds were also tested for toxicity at different temperatures. For this test use was made of the warm (and cold) microscope stage, and both culture and toxic solution were kept immersed in baths at the same temperature as the stage. The results are given in Table IV.

Table IV. *Toxic concentration at various temperatures.*

| Substance | 9·5° | 15·5° | 17·5° | 22·0° | 27·0° |
|---|---|---|---|---|---|
| Mercuric chloride | *M*/22,500 | — | *M*/24,300 | *M*/27,000 | — |
| Nickel chloride | *M*/475 | — | *M*/583 | *M*/667 | — |
| *p*-Chlorophenol | — | *M*/317 | — | — | *M*/317 |
| Formaldehyde | — | *M*/262 | — | — | *M*/283 |

It will be seen that increased temperature was accompanied by decreased resistance on the part of the organisms. The variation in the case of nickel chloride amounted to some 33 % and in the case of mercuric chloride to some 18 %, both over a range of 12·5°. Clearly the slight variations in temperature (1° to 2°) experienced under normal conditions could have exerted no appreciable effect upon the toxicity measurements.

*Variation in hydrogen ion concentration.* Variations in $p_H$ may affect toxicity measurements either by influencing the rate of penetration of the toxic agent or by affecting the organisms directly. The former possibility has been subjected to investigation in recent years. Brooks [1925] has examined the effect of $p_H$ upon the penetration of arsenic into living cells (*Valonia*) and Crane [1921] has shown that the toxicity of certain alkaloids to *P. caudatum* varies with the $p_H$ attributable to variations in the degree of dissociation of the alkaloidal salt. Table V shows the effect of variation in $p_H$ upon the toxicity of quinine sulphate to *Colpidium* and serves to confirm Crane's conclusion.

Table V. *Toxicity of quinine sulphate.*

| $p_H$ | 7·7 | 7·4 | 6·9 | 6·4 | 5·9 |
|---|---|---|---|---|---|
| Toxic concentration | *M*/1438 | *M*/970 | *M*/930 | *M*/890 | > *M*/890 |

The effect of $p_H$ upon the organisms themselves has been investigated by Dale [1913], who showed that *Paramecium* cannot live in a solution more acid than $p_H$ 5·0 for more than 5 minutes. Experiment showed that *Colpidium* could only live within the limits $p_H$ 5·0 to 9·5. Most of the substances tested for toxicity in this investigation have been presented in amounts too small to affect the $p_H$ to any significant extent.

*Contamination of cultures.* The possibility of toxicity measurements being affected by the presence of some foreign organism was not considered in the above standardisation, for the culture showed no evidence of bacterial growth. It was not until a grossly infected culture chanced to be used, giving markedly different toxicity figures, that the importance of contamination was realised. The effect of contamination was always to reduce the toxicity of the substance tested. There can be little doubt that this effect is due entirely to the presence of débris, resulting from bacterial growth. In the course of this work many contaminated cultures have been encountered and the application of different forms of filtration has made it quite clear that it was the débris that was affecting the toxicity figures, presumably by absorbing a significant amount of the toxic agent. So long as the culture remained clear, although bacteriologically contaminated, it gave consistent results. In this respect *Glaucoma scintillans* has proved superior to *C. colpoda*, having maintained its clarity throughout, over a period of 2 years. A similar effect has been observed by Morgan and Cooper [1924, 1] and Cooper *et al.* [1925]; they state that the bactericidal power of quinones and certain other compounds is greatly reduced by the presence of organic matter.

None of the above sources of error appeared to be serious and it was felt that, provided cultures were used about the time of their maximum growth, reliable toxicity measurements could be made, in which the margin of error would not exceed 40 % and would probably be less. The following tables contain the results of the examination of a large number of arsenic compounds and a lesser number of miscellaneous compounds, reputed to be toxic. In the first column, opposite each compound, is given the toxicity figure, expressed in the molecular concentration which kills in 3 minutes. In the second column, a figure is given expressing the toxicity relative to the arbitrary toxic value of 100 given to diphenylchloroarsine, which was adopted as a standard. Corresponding figures are also given for the toxicity to *G. scintillans* wherever tests have been carried out with that organism. It should be noted that the symbol $>$, when used in the first column, means that the *toxic concentration* is greater than the figure given; the actual toxicity of that compound is therefore *less* than the figure given in the second column.

It has been impossible to include in these tables all the compounds examined. Those given should be regarded as representative of a far larger number in each series, a fact which has been taken into account when formulating conclusions.

## *Tervalent arsenic compounds.*

| Name | Toxicity to *Colpidium*: Concentration which kills in 3 mins. | Toxicity to *Colpidium*: Toxicity relative to diphenylchloroarsine =100 | Toxicity to *Glaucoma*: Concentration which kills in 3 mins. | Toxicity to *Glaucoma*: Toxicity relative to diphenylchloroarsine =100 |
|---|---|---|---|---|
| *Primary derivatives:* | | | | |
| *p*-Chlorophenyldichloroarsine | *M*/3300 | 2 | — | — |
| *o*-Nitrophenyldichloroarsine | >*M*/4460 | <2 | *M*/5870 | 1 |
| *o*-Bromophenyldichloroarsine | *M*/5460 | 3 | *M*/23,470 | 6 |
| Phenylarsenious oxide | >*M*/9160 | <5 | *M*/16,000 | 4 |
| *o*-Benzarsenious oxide | >*M*/50 | 0 | — | — |
| Benzophenone-*o*-arsenious oxide | >*M*/42,000 | <23 | — | — |
| Benzophenone-*o*-arsenious chloride | *M*/1100 | 1 | — | — |
| *o*-Hydroxyphenylarsenious oxide anhydride | >*M*/11,000 | <6 | — | — |
| Methyldichloroarsine | >*M*/2000 | 0 | >*M*/2000 | 0 |
| Butyldichloroarsine | *M*/900 | 1 | — | — |
| *Secondary derivatives:* | | | | |
| Diphenylchloroarsine | *M*/180,000 | 100 | *M*/420,000 | 100 |
| Diphenylbromoarsine | *M*/228,000 | 127 | — | — |
| Diphenylcyanoarsine | *M*/182,700 | 101 | — | — |
| Diphenylarsenious sulphide | *M*/2100 | 1 | — | — |
| Diphenylarsenious oxide | *M*/224,000 | 124 | — | — |
| 10-Cyanophenoxyarsine | *M*/261,300 | 145 | *M*/466,200 | 117 |
| 10-Chloro-5 : 10-dihydrophenarsazine | *M*/102,000 | 57 | *M*/166,500 | 42 |
| 10-Bromo-5 : 10-dihydrophenarsazine | *M*/71,500 | 40 | *M*/151,500 | 38 |
| 10-Iodo-5 : 10-dihydrophenarsazine | *M*/80,000 | 44 | *M*/69,000 | 17 |
| 10-Aceto-5 : 10-dihydrophenarsazine | *M*/256,300 | 142 | *M*/493,300 | 123 |
| 10 : 10-Sulpho-5 : 10-dihydrophenarsazine | >*M*/15,000 | <8 | >*M*/15,000 | <4 |
| 10 : 10-Oxy-5 : 10-dihydrophenarsazine | *M*/115,000 | 64 | *M*/332,500 | 83 |
| 10 : 10-*Bis*-5 : 10-dihydrophenarsazine | *M*/40,000 | 22 | *M*/57,400 | 14 |
| 5-Acetyl-10-chloro-5 : 10-dihydrophenarsazine | *M*/21,000 | 12 | *M*/45,560 | 11 |
| 5-Propionyl-10-chloro-5 : 10-dihydrophenarsazine | *M*/17,400 | 10 | *M*/34,140 | 8 |
| 3-Amino-10-chloro-5 : 10-dihydrophenarsazine hydrochloride | *M*/16,000 | 9 | *M*/33,000 | 8 |
| 2-Amino-10-chloro-5 : 10-dihydrophenarsazine hydrochloride | *M*/10,000 | 6 | *M*/14,400 | 4 |
| 3-Carboxy-10-chloro-5 : 10-dihydrophenarsazine | >*M*/50,000 | <28 | >*M*/50,000 | <12 |
| 4-Carboxy-10-chloro-5 : 10-dihydrophenarsazine | >*M*/16,000 | <9 | >*M*/16,000 | <4 |
| 2 : 10-Dichloro-5 : 10-dihydrophenarsazine | *M*/21,000 | 12 | *M*/34,000 | 8 |
| 3 : 10-Dichloro-5 : 10-dihydrophenarsazine | >*M*/75,000 | <42 | *M*/92,000 | 48 |
| 2 (or 8)-Methyl-10-chloro-5 : 10-dihydrophenarsazine | *M*/110,000 | 61 | *M*/320,000 | 80 |
| *N*-Acetyl-2-(or 8)-methyl-10-chloro-5 : 10-dihydrophenarsazine | *M*/36,000 | 20 | *M*/102,300 | 26 |
| 2 : 8-Dimethyl-10-chloro-5 : 10-dihydrophenarsazine | >*M*/120,000 | <67 | >*M*/120,000 | <30 |
| 12-Chloro-12 : 7-dihydrobenzophenarsazine | >*M*/100,000 | <55 | >*M*/100,000 | <25 |
| 10-Methyl-12-chloro-12 : 7-dihydrobenzophenarsazine | >*M*/100,000 | <55 | >*M*/100,000 | <25 |
| 7-Chloro-7 : 14-dihydrodibenzophenarsazine | >*M*/100,000 | <55 | >*M*/100,000 | <25 |
| 14-Chloro-7 : 14-dihydrodibenzophenarsazine | >*M*/50,000 | <28 | >*M*/50,000 | <13 |
| Phenylchloroarsine acetic acid | *M*/160 | 0 | — | — |
| Phenylethylmethylchloroarsine | *M*/12,700 | 7 | — | — |
| Phenylethoxychloroarsine | >*M*/4800 | <3 | *M*/13,000 | 3 |
| Cacodyl chloride | *M*/2260 | 1 | — | — |
| Cacodyl iodide | *M*/2260 | 1 | — | — |
| Cacodyl oxide | *M*/1550 | 1 | — | — |
| Cacodyl cyanide | *M*/47 | 0 | — | — |

## *Quinquevalent arsenic compounds.*

| | | | | |
|---|---|---|---|---|
| *Primary derivatives:* | | | | |
| Diphenyl-4 : 4′-diarsinic acid | *M*/4000 | 2 | *M*/4000 | 1 |
| *o*-Benzarsinic acid | *M*/140 | 0 | — | — |
| *o*-Tolylarsinic acid | *M*/450 | 0 | — | — |
| *o*-Arsanilic acid | *M*/570 | 0 | — | — |
| Benzophenone-*o*-arsinic acid | *M*/920 | 1 | — | — |
| Azobenzene-*o* : *o*′-diarsinic acid | >*M*/3500 | <2 | — | — |
| *Secondary derivatives:* | | | | |
| Diphenylarsinic acid | *M*/1500 | 1 | — | — |
| α-Naphthylarsinic acid | *M*/620 | 0 | *M*/850 | 0 |
| *o* : *o*′-Dicarboxydiphenylarsinic acid anhydride | >*M*/11,000 | <6 | — | — |
| *Tertiary derivatives:* | | | | |
| Triphenylarsine dichloride | *M*/670 | 0 | — | — |

*Antimony compounds.*

| Name | Toxicity to *Colpidium*: Concentration which kills in 3 mins. | Toxicity to *Colpidium*: Toxicity relative to diphenylchloroarsine =100 | Toxicity to *Glaucoma*: Concentration which kills in 3 mins. | Toxicity to *Glaucoma*: Toxicity relative to diphenylchloroarsine =100 |
|---|---|---|---|---|
| Diphenylchlorostibine | *M*/183,000 | 101 | — | — |
| Triphenylstibine dichloride | *M*/700 | 0 | — | — |
| Dimethylbromostibine | *M*/3400 | 2 | — | — |
| Trimethylstibine dichloride | *M*/1140 | 1 | — | — |
| Trimethylstibine hydroxybromide | >*M*/80 | 0 | — | — |

*Mercury compounds.*

| | | | | |
|---|---|---|---|---|
| Phenyl mercuric chloride | *M*/1,152,000 | 640 | — | — |
| Allyl mercuric iodide | *M*/599,000 | 333 | *M*/442,200 | 110 |

*Miscellaneous compounds.*

| | | | | |
|---|---|---|---|---|
| Diphenylbromobismuthine | *M*/270,000 | 150 | — | — |
| Triphenylbismuthine | *M*/11,000 | 6 | — | — |
| Tri-*p*-tolylbismuthine | >*M*/2000 | <1 | — | — |
| Triphenylbismuthine difluoride | *M*/7100 | 4 | — | — |
| Methyl chloroacetate | *M*/8 | 0 | — | — |
| Chloromethyl chloroacetate | *M*/300 | 0 | *M*/400 | 0 |
| Chloromethyl trichloroacetate | *M*/240 | 0 | *M*/400 | 0 |
| Trichloromethyl trichloroacetate | >*M*/550 | 0 | >*M*/550 | 0 |
| *m*-Methoxybenzyl bromide | *M*/1560 | 1 | — | — |
| Dicyanofuroxan | *M*/800 | 0 | *M*/950 | 0 |
| Allyl isothiocyanate | *M*/100 | 0 | — | — |
| Hexachlorodimethyl carbonate | *M*/5000 | 3 | *M*/4400 | 1 |
| Chloroaceto-*p*-bromoanilide | >*M*/2500 | <1 | >*M*/2500 | <1 |

## Discussion.

Before proceeding to discuss the results detailed in the preceding tables, it should be mentioned that a preliminary investigation was carried out in order to ascertain whether the protozoal test gave results concordant with the general knowledge available concerning toxicity. That investigation is reported elsewhere [Walker, 1922].

The results, for both organic and inorganic compounds, indicated that unicellular organisms provide a useful means of measuring general protoplasmic toxicity. The protozoal test, however, fails to give any indication of poisons exerting a specific action upon mammals; for example, the cyanides, certain amines and the alkaloids.

### *Consideration of present results.*

The present results afford striking confirmation of the conclusions reached by McCleland and Peters [1919]. These conclusions were "for similar compounds:

(*a*) primary compounds are less toxic than secondary,

(*b*) aliphatic compounds are less toxic than aromatic,

(*c*) arsenic compounds are less toxic than antimony."

With regard to conclusion (*a*) the evidence in its favour brought forward here is overwhelming. Conclusion (*b*) is amply supported and is in accordance with the general rule concerning aliphatic and aromatic compounds. The

evidence in favour of conclusion (*c*) is definite but too slight to render a sweeping assertion possible. McCleland and Peters, however, only examined tervalent derivatives of arsenic. In the present work a large number of both ter- and quinque-valent arsenic derivatives have been examined, with the result that it is possible to make a fourth generalisation; viz. tervalent derivatives are more toxic than quinquevalent derivatives of arsenic. This generalisation is commonly implied in the explanation of the trypanocidal action of quinquevalent arsenic compounds; for such compounds are but slightly toxic to trypanosomes *in vitro* and it is assumed that they are reduced *in vivo* to the tervalent state prior to exerting their trypanocidal action [Durham, Marchal and King, 1926; Voegtlin *et al.*, 1923]. The number of tertiary derivatives of arsenic which have been examined is small, but if, as appears to be justified, the results for antimony compounds be taken into account as well, it will be seen that yet a fifth generalisation is possible; viz. that tertiary derivatives are less toxic than secondary derivatives of arsenic. The evidence also suggests that tertiary are less toxic than primary derivatives. Thus, there are four conclusions well established:

1. Aromatic derivatives are more toxic than aliphatic derivatives of arsenic.

2. Tervalent derivatives are more toxic than quinquevalent derivatives of arsenic.

3. Secondary derivatives are more toxic than primary and tertiary derivatives of arsenic.

4. Primary derivatives are more toxic than tertiary derivatives of arsenic. Whilst the evidence in favour of a fifth is suggestive.

5. Antimony compounds are more toxic than the corresponding arsenic compounds.

There is little doubt that conclusions 1–4 are as applicable to antimony (and probably bismuth) as to arsenic. Conclusion 2 may be expressed in another manner: amongst arsenic compounds the greater the susceptibility to oxidation, the greater the toxicity. Whether such conclusion is capable of extension to other metals is at least an open question; Morgan and Cooper [1924, 1] state that tellurium in bivalent combination is far superior to quadrivalent tellurium as a bactericidal agent.

An outstanding feature of the results is the high toxicity of tervalent secondary derivatives of arsenic; and within this class those compounds in which the arsenic atom is associated with the diphenyl group (or some modification of it) are characterised by the highest toxicity. So long as the arsenic is linked up with the diphenyl group it seems to be of little significance what element or group engages the third valency of the arsenic, provided that such group is capable of being readily replaced by an hydroxyl group—an important point. Thus diphenylchloroarsine, diphenylbromoarsine, diphenylcyanoarsine and diphenylarsenious oxide are all of the same high order of toxicity—and all can, in aqueous medium, give rise to diphenylarsenious

hydroxide. Linking up the diphenyl groups alters the toxicity but little. This is best illustrated by the examples of diphenylchloroarsine, $C_6H_5.AsCl.C_6H_5$, $M$/180,000, cyanophenoxyarsine, $C_6H_4\langle^{O}_{AsCN}\rangle C_6H_4$, $M$/261,300, and 10-chloro-5 : 10-dihydrophenarsazine, $C_6H_4\langle^{NH}_{AsCl}\rangle C_6H_4$, $M$/102,000.

The above examples form an interesting group, from which it appears that the exact type of linkage of the two phenyl groups has but little effect upon the high toxicity of these compounds. But any other modification of the structure inevitably leads to a fall in toxicity, in some cases very marked. Thus, substitution of one or both phenyl groups by naphthyl groups leads to a fall in toxicity, and substitution of one or both phenyls by aliphatic radicles again leads to a fall in toxicity. Substitution in the benzene ring at once reduces the toxicity; this has been tested by the insertion in the ring of the following groups: Cl, —COOH, $—NH_2$ and $—CH_3$. Ample evidence in support of the above statements is to be found in the tables.

As already mentioned, the toxicity seems but little affected by the third group attached to the arsenic, provided it is hydrolysable; for there can be little doubt that these compounds, administered as they are in such dilute solution, exert their toxic effect in the hydrolysed form. The rate of hydrolysis of diphenylchloroarsine, for instance, is described by Rona [1921] as "momentan." It may be mentioned that there can be no question of acid effect in these toxicity measurements, since there was always more than enough pyridine present to neutralise the acid liberated. Further proof that these compounds exert their toxic action in the hydroxide form is that the corresponding oxides are aH rather more toxic than the halogen derivatives, as would be expected since one molecule of the oxide can give rise to two molecules of the hydroxide:

$$[(C_6H_5)_2As]_2O \xrightarrow{H_2O} 2(C_6H_5)_2As\text{-}OH.$$

Such variations in toxicity as occur among a series of diphenylarsine derivatives, in which the third hydrogen atom is replaced by different groups (Cl, Br, I, CN, O), are probably due to concomitant variations in solubility and rate of hydrolysis. One further point concerning the secondary derivatives of arsenic is worthy of note: all these highly toxic compounds are readily lipoid-soluble and but slightly soluble in water. Whether this is an essential condition to high toxicity or whether it is merely coincidental is a matter of opinion.

The mercury compounds examined are of considerable interest in view of their general high toxicity. The substitution of a phenyl (or allyl) radicle for one of the chlorine atoms of mercuric chloride enhances the toxicity enormously. It has already been noted that the highly toxic arsenic compounds are all readily lipoid-soluble and it seems probable that these organic derivatives of mercury owe their enhanced toxicity (over mercuric chloride)

mainly to their ready solubility in lipoids. It is not suggested that lipoid-solubility is the only factor influencing toxicity in this series of compounds, but the absence of information concerning the precise mode of action of mercury compounds prevents the appreciation of whatever other factors are at work.

Amongst the miscellaneous compounds examined, those containing bismuth suggest that this metal ranks with arsenic in toxicity, whilst tellurium belongs to a lower order of toxicity. The remaining miscellaneous compounds were tested because they have been reported, at different times, in the current literature as possessing toxic properties. It will be seen that they are generally of a low order of toxicity, at least as protoplasmic poisons, and no feature of interest is raised by them.

### *Application of results to higher animals.*

Many of the compounds examined for toxicity to protozoa, particularly the secondary directives of arsenic, are known to be powerful respiratory irritants. It is a matter of some interest therefore to ascertain whether any relationship exists between toxicity as measured against protozoa and respiratory irritant power.

The work of Flury [1921] and of Tattersfield and Roberts [1920] suggests that such a relationship does exist and the results recorded in this paper point to a similar conclusion. There are, however, notable exceptions which prevent the promulgation of the simple rule that toxicity to *Colpidium* is a measure of irritancy. The question cannot be discussed here, but this much may be said: if it be permitted to suppose that high (sensory) irritant power resides in an optimum combination of

(*a*) a primary irritant action (directly upon nerve endings), and

(*b*) a weak but prolonged irritant action (indirectly, through the swelling of protoplasm and consequent pressure upon nerve endings), then all the results are capable of interpretation.

Toxicity to protozoa would give a measure solely of (*b*) and therefore would give, when high enough, an apparent correspondence between toxicity to *Colpidium* and sensory irritant power.

### *Mode of action of arsenicals.*

The precise mode of action of arsenic in its various combinations has been investigated intermittently for many years and throughout there appears to have been an appreciation of the significance of the two states, the ter- and the quinque-valent, in which arsenic can exist. The pioneer work in this field was done by Ehrlich and Hata [1910], particularly in relation to therapeutical measurements. A brief résumé of the position is to be found in a report by Voegtlin *et al.* [1923]. Attempts have been made [Rona *et al.*, 1922; Flury, 1921] to show that arsenic owes its intrinsic toxicity to the power of inhibiting enzyme action, but little success has attended these efforts. The

most substantial advance is that made by Voegtlin *et al.* [1923], who have presented a very strong case for the view that arsenic specifically attacks the sulphydryl group of the cell (and, in particular, reduced glutathione). In the course of this present investigation it has been fully demonstrated that, of all the arsenic compounds, the tervalent secondary derivatives are the most toxic. It was observed that all these highly toxic compounds seemed to possess the common property of being susceptible to oxidation, even to atmospheric oxidation. This property is best demonstrated by dissolving the compound in turpentine; if a trace of water be added to ensure hydrolysis, the corresponding arsinic acid crystallises out in the course of an hour or two. An account of a typical case of this oxidation is given elsewhere [Walker, 1925]. This case of oxidation suggested that this type of arsenic compound might interfere in the oxidation-reduction system of the cell. Accordingly, the effect of a typical compound (diphenylchloroarsine) upon the washed muscle—glutathione system of Dixon and Hopkins [1922]—was investigated. Evidence was obtained of a definite interference with the oxidation of the tissue, attributable to an inhibition of the glutathione by the arsenic. It was difficult to give precision to this line of investigation, owing to the very low solubility of diphenylchloroarsine in water. Recourse was therefore had to a direct investigation of the effect of sulphydryl compounds upon the toxicity of diphenylchloroarsine to protozoa. To this end, the effect was examined of varying concentrations of sodium thioglycollate upon the toxic effect of a solution of diphenylchloroarsine. The sulphydryl compound was added to the solution of arsenic compound prior to the addition of the latter to the culture in the normal way. Table VI contains the results; the two concentrations refer to one and the same solution.

Table VI.

| Concentration of diphenylchloroarsine mg. per litre | Concentration of sodium thioglycollate mg. per litre | Time required to kill all the organisms mins. |
|---|---|---|
| 2·5 | 0 | 9½ |
| 2·5 | 2·5 | 24 |
| 2·5 | 6·25 | 33 |
| 2·5 | 12·5 | 62 |
| 2·5 | 25·0 | 175 |
| 2·5 | 50·0 | >180 |

This illustrates the very marked detoxicating effect of a simple sulphydryl compound upon diphenylchloroarsine. The investigation was extended further to show that the organisms could be made to survive considerable periods (30–60 minutes), by the addition of a small amount of sodium thioglycollate 2 minutes after the addition to the culture of a dose of diphenylchloroarsine which was lethal normally in 3 minutes. Finally it was found that by the addition of a few drops of a suitable sulphydryl solution, at the end of 3 minutes, to a culture which had received a 3-minute lethal dose of arsenic, the organisms could be revived. The sulphydryl solution was added immediately

all movement of the organisms had stopped; revival was rapid and in one minute some 90 % of the organisms were rapidly swimming about again. Death took place ultimately after 1–2 hours. These are the only circumstances in which the organisms have been seen to resume movement after having once stopped under the influence of an arsenic compound. For this last test the sulphydryl compound used was monothioethylene glycol, prepared by Bennett's [1922] method. A 0·1 % solution in water, neutralised, was used. This compound is superior to most sulphydryl compounds in being more stable and in being readily soluble both in water and organic solvents (and therefore presumably lipoid-soluble). It is clear, therefore, that sulphydryl compounds possess the power of counteracting the toxic effect of arsenic compounds to a marked degree and it is of interest that this conclusion was reached independently, and by a different route, to the similar conclusion of Voegtlin.

Whatever significance may be attached to this result it would appear that the susceptibility of the tervalent arsenic compounds to oxidation can only be regarded as a measure of the combining power of the compound, an indication of the potential of the arsenic in the molecule. For it is in the molecule as a whole that the toxicity resides, not in the arsenic alone, or in the constituent groups. The radicles attached to the arsenic are only important in so far as they affect the reactivity of the arsenic and it seems probable that in the diphenylarsine structure the optimum degree of reactivity is to be found.

## Summary.

1. The relationship between chemical constitution and toxicity has been studied, toxicity being measured against the unicellular organism, *Colpidium colpoda*, and, to a lesser extent, against *Glaucoma scintillans*.

2. The bulk of the compounds examined are derivatives of arsenic; a lesser number of antimony, bismuth and mercury compounds have also been examined.

3. The following general rules, appertaining to arsenic compounds, have been established:

(*a*) aromatic derivatives are more toxic than aliphatic derivatives;
(*b*) tervalent derivatives are more toxic than quinquevalent derivatives;
(*c*) secondary derivatives are more toxic than primary and tertiary derivatives;
(*d*) primary derivatives are more toxic than tertiary derivatives.

4. There is suggestive evidence that antimony compounds are more toxic than the corresponding arsenic compounds.

5. The most toxic arsenic compounds are the tervalent secondary derivatives and, within this class, compounds of the diphenylarsine type of structure are the most toxic.

6. It is considered that unicellular organisms only afford a measure of general protoplasmic toxicity.

7. Evidence is brought forward to show that the toxicity of arsenicals is intimately concerned with the sulphydryl constituents of the cell. Organisms which have ceased all movement under the influence of arsenic can be revived at once, and continue to live for periods up to 2 hours, by the addition of a suitable sulphydryl compound.

In conclusion I should like to record my gratitude to Prof. R. A. Peters for his advice and interest throughout this work.

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